

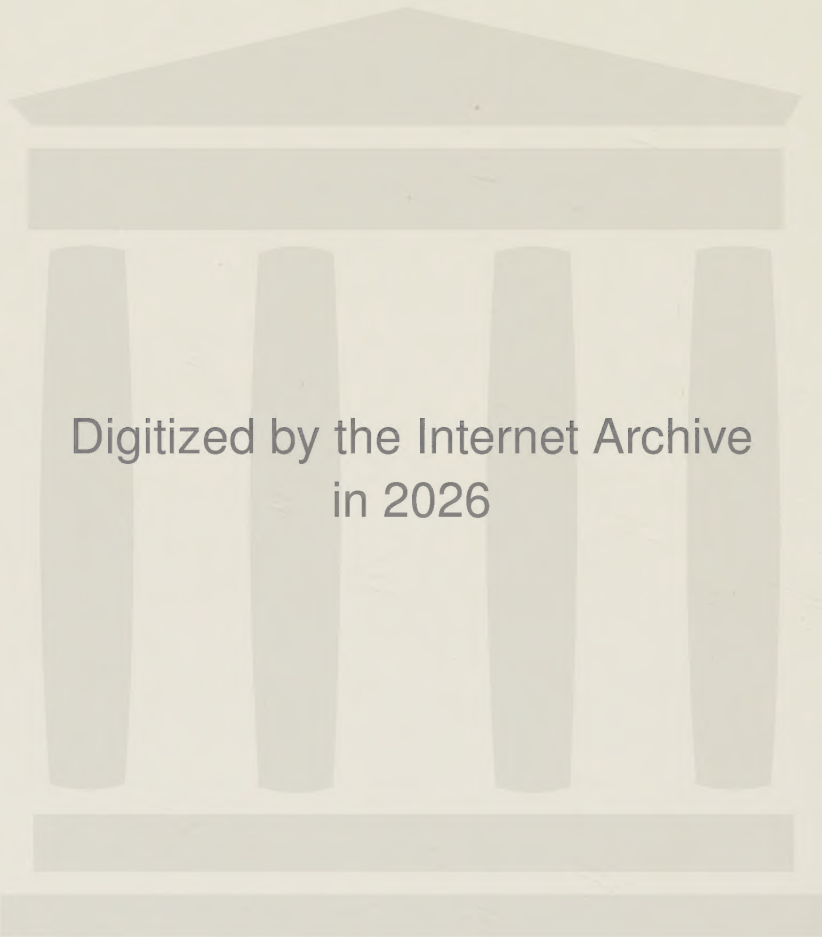
DIAMINE AND POLYAMINE METABOLISM IN MAMMALIAN REPRODUCTION

An experimental study in the female rat

By

Anne-Charlotte Henningsson

Lund 1981



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To Stig,
Lea and Drutten



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The present thesis is based mainly on the following publications which in the text will be referred to by their Roman numerals.

- I. ANDERSSON, A.-CH., HENNINGSSON, S. & ROSENGREN, E. (1978). Increased formation of diamines and polyamines in the pregnant rat. *J. Physiol.* 285: 311-324.
- II. ANDERSSON, A.-CH. & HENNINGSSON, S. (1981). On the biogenesis of diamines and polyamines in the pregnant rat. *Acta Endocrinol.* In press.
- III. ANDERSSON, A.-CH., HENNINGSSON, S. & ROSENGREN, E. (1979). Formation of cadaverine in the pregnant rat. *Acta Physiol. Scand.* 105: 508-512.
- IV. ANDERSSON, A.-CH. & HENNINGSSON, S. (1980). Biosynthesis and accumulation of cadaverine and putrescine in rat ovary after administration of human chorionic gonadotrophin. *Acta Endocrinol.* 95: 237-243.
- V. ANDERSSON, A.-Ch., HENNINGSSON, S., PERSSON, L. & ROSENGREN, E. (1978). Aspects on diamine oxidase activity and its determination. *Acta Physiol. Scand.* 102: 159-166.
- VI. ANDERSSON, A.-CH. & HENNINGSSON, S. (1981). Metabolism of putrescine in the pregnant rat. *Acta Physiol. Scand.* In press.

CONTENTS

INTRODUCTION	7
METHODOLOGY	14
RESULTS AND COMMENTS	21
Urinary excretion of diamines and polyamines in the pregnant rat	21
Formation of cadaverine in the pregnant rat	28
Diamine and polyamine metabolism in rat ovary after administration of human chorionic gonadotrophin	29
Determination of tissue diamine oxidase activity	32
In vivo metabolism of putrescine in the pregnant rat	35
Content of diamines and polyamines in body fluids and tissues of the pregnant rat	37
DISCUSSION	38
SUMMARY AND CONCLUSIONS	46
REFERENCES	48
APPENDIX: Publications I-VI	61

INTRODUCTION

The ability of a tissue to inactivate a diamine was originally demonstrated by Eustis (1915) who noted that histamine lost its biological effect on incubation with a liver extract from the turkey buzzard. The phenomenon, soon shown to occur in other tissues as well (Best 1929), was assumed to be due to enzymatic inactivation of histamine and the enzyme responsible was named histaminase (Best & McHenry 1930). Histaminase was shown to act not specifically on histamine but also on other diamines such as putrescine, cadaverine and agmatine and the enzyme was proposed to be properly called diamine oxidase (Zeller 1938; 1942; 1965). Histaminase is now generally considered identical with diamine oxidase.

Pregnancy was found to be a physiological condition closely related to elevated histaminase levels as the placenta (Danforth & Gorham 1937; Zeller, Schär & Staehlin 1939; Ahlmark 1944; Swanberg 1950) and the blood (Marcou, Athanasiu-Vergu, Chiricéanu, Cosma, Gingold & Parhon 1938) of pregnant women were found to possess a strong histaminolytic property. These findings were followed by numerous investigations where the plasma diamine oxidase level during the course of pregnancy was measured (Werle & Effke-mann 1940; Anrep, Barsoum, Ibrahim & Amin, 1941; Anrep, Barsoum & Ibrahim 1947a) and correlations between the plasma level and the diagnosis of pregnancy, the prognosis of threatening abortion (Zeller & Birkhäuser 1940; Labhardt 1941; Walentin 1945; Genell 1950; Borglin & Willert 1957) as well as other obstetrical conditions (Kapeller-Adler 1944; Anrep, Barsoum & Ibrahim 1947b; Borglin & Willert 1961) were made. The hope was excited that measurements of plasma diamine oxidase levels would be clinically valuable as an index of fetoplacental functionality and that fetal salvage programmes might be based on assays of the enzyme (Weingold & Southren 1968; Weingold, Southren & Lee 1971). Some authors, however have presented critical views on the value of plasma diamine oxidase determinations in pregnancy as they found that the diamine oxidase values during normal pregnancy could vary over a rather wide range (Resnik & Le-

vine 1969; Ward, Rochman, Varnavides & Whyley 1973; Scott, Childs, Crabbe, Tindall & Bardsley 1979). Recently measurement of diamine oxidase activity in plasma during early pregnancy for prediction of date of confinement (Elmfors & Tryding 1976) and in vaginal fluid for the diagnosis of ruptured fetal membranes (Elmfors, Tryding & Tufvesson 1974) was found to be techniques suitable for routine clinical use.

Pregnancy was first considered to be the only condition associated with elevated levels of diamine oxidase but it was later shown that certain clinical conditions such as treatment with heparin (Tryding 1965) and carcinomas of various kinds (Benda & Miravet 1960; Borglin & Willert 1962; Baylin, Beaven, Engelman & Sjoerdsma 1970; Baylin, Abeloff, Wieman, Tomford & Ettinger 1975; Lin, Orcutt & Stohlbach 1975) were connected with changes in diamine oxidase activity.

In the early reports on diamine oxidase and reproduction elevated levels of this enzyme were thought to occur only in human pregnancy. However, Ahlmark (1944) showed a strongly increased histaminolytic power of placental tissue and plasma of several species, e.g. rat and guinea-pig, during pregnancy. These findings in the rat were later confirmed and extended (Roberts & Robson 1953; Kobayashi 1964) and elevated diamine oxidase levels were also shown in the liver (Zeller 1940a) as well as in several other tissues (Kim & Harris 1973) of the pregnant rat.

In the measurement of diamine oxidase activity *in vitro* various methods and substrates have been used. These methods include determinations of oxygen consumption (Gebauer-Fuellnegg & Alt 1932; Zeller, Birkhäuser, Mislin & Wenk 1939), production of ammonia (Zeller 1940b), production of hydrogen peroxide (Zeller 1940a; 1941) and determination of the remaining amount of substrate after the incubation with histamine (Best 1929; Edlbacher & Zeller 1937). Using β -³H-histamine as a substrate determinations of the tritiated water produced has been used as a measure of diamine oxidase activity (Beaven & Jacobsen 1971).

With putrescine as substrate for diamine oxidase, the product of the reaction, γ -aminobutyraldehyde, is non-enzymic

matically transformed into the cyclic compound Δ^1 -pyrroline (Tabor 1951; Hasse & Maisack 1955; Mann & Smithies 1955) which in the presence of o-aminobenzaldehyde condenses to form a coloured complex that is measured spectrophotometrically (Holmstedt & Tham 1959). When ^{14}C -putrescine is employed as substrate the radioactive Δ^1 -pyrroline formed is extracted and used as a measure of diamine oxidase activity (Okuyama & Kobayashi 1961). This method, based on determinations of one metabolite of ^{14}C -putrescine, stimulated renewed interest in plasma diamine oxidase activity during pregnancy (Kobayashi 1963; Southren, Kobayashi, Sherman, Levine, Gordon & Weingold 1964; Resnik & Levine 1969; Ward et al. 1973; Elmfors et al. 1974; Elmfors & Tryding 1976). However, in vivo studies, involving the administration of ^{14}C -putrescine to rats and mice, have shown the occurrence of a further metabolism of γ -aminobutyraldehyde. Thus radiolabelled putrescine carbon has been shown both to be incorporated into γ -aminobutyric acid (GABA) (Seiler, Wiechmann, Fischer & Werner 1971; Seiler & Al-Therib 1974; Henningsson & Rosengren 1976) and excreted as $^{14}\text{CO}_2$ (Jänne 1967; Seiler & Eichenkopf 1975; Henningsson & Rosengren 1976). Among the ^{14}C -metabolites excreted in the urine after the injection of ^{14}C -putrescine some unidentified compound(s) could be found in great amounts (Seiler & Eichenkopf 1975; Henningsson & Rosengren 1976).

The pregnant rat, in spite of the elevated levels of diamine oxidase in plasma and various organs, excreted large amounts of histamine in the urine during the last third of pregnancy (Kahlson, Rosengren & Westling 1958a; Angervall, Enerbäck & Westling 1960). The excreted amounts were shown to be larger the greater the number of young in the litter, suggesting an importance of the presence of the fetuses for the elevated amounts of histamine appearing in the urine of the mother (Kahlson et al. 1958a). This view was supported by experiments showing that after removal of the fetuses before term the increased urinary excretion of histamine was rapidly abolished (Kahlson, Rosengren, Westling & White 1958b). Further, in vitro experiments revealed a high rate of fetal histamine formation (Kahlson et al. 1958b). Particularly the livers of the fetuses exhibited a high

ability of forming histamine (Kahlson, Rosengren & White 1960; Telford & West 1961; Burkhalter 1962). The increased histamine formation of the fetuses as reflected in the urinary excretion of the mother roughly corresponded to the period of rapid increase in weight of the fetuses occurring after the 15th day of pregnancy.

The female hamster, likewise excreted elevated amounts of histamine in the urine during the last third of pregnancy (Rosengren 1965). However, the histamine formation of the fetuses was rather low. In this species the placenta was likely to account for the excessive amounts of urinary histamine occurring during pregnancy as it was found to possess a high ability to form histamine in vitro.

At variance with the situation in the rat and hamster, the mouse excreted increased amounts of histamine in the urine from the very first day of pregnancy, ascribed to an increased formation of the amine in the kidneys of the mother. Further, the fetuses of the mouse, like the fetuses of the rat formed histamine in vitro, though the main capacity of histamine formation of the mouse fetus was located to the skin (Rosengren 1963; Maudsley & West 1964).

Analogous to the findings in rodents, an increased urinary histamine excretion has been found in human pregnancy (Bjurö, Lindberg & Westling 1961). Granerus, Gillbrand & Wetterqvist (1977) confirmed an increased histamine turnover in human pregnancy taking into consideration also the major histamine metabolites in man and the dietary conditions which were standardized. Histamine formation of the growing human fetus was disclosed by the finding of a higher histamine level in the umbilical artery plasma than in the plasma of the umbilical vein (Kahlson, Rosengren & White 1959) as well as by isotopic in vitro determinations of histidine decarboxylase activity (Lindberg, Lindell & Westling 1963a).

The physiological significance of histamine during pregnancy was suggested to be related to processes of rapid growth (Kahlson et al. 1960; Kahlson 1962). However, the role of histamine as a growth regulating factor has remained elusive and the interest in this respect has been focused on the diamine putrescine and the polyamines spermi-

dine and spermine, low molecular aliphatic bases which appear to be ubiquitously present in animal, microbial as well as plant material.

The discovery of a polyamine dates back to the late seventeenth century when Lewenhoeck (1678) observed the deposition of some characteristic crystals in a human seminal sample. A century later, these crystals, shown to be identical with crystals obtained from many tissues (Schreiner 1878), received the name spermine (Ladenburg & Abel 1888). Whilst performing synthetic work on spermine another base, identified as spermidine (Dudley, Rosenheim & Starling 1927), was obtained. The polyamine precursor putrescine and its close congener cadaverine were first detected by Brieger (1885) in putrefying animal tissue. Subsequently, there were many reports on the isolation of these diamines from material undergoing bacterial decomposition (for ref. see Guggenheim 1951). For several years it was believed that the occasional finding of diamines and polyamines in higher organisms was related to bacterial contamination or bacterial decay processes. However, after the discovery of an accumulation of polyamines in chick embryo during development (Raina 1963) and in rat liver after partial hepatectomy (Dykstra & Herbst 1965) this view was reconsidered.

One of the first reports suggesting a physiological importance of putrescine and polyamines was the observation that these amines were essential growth factors for *Hemophilus parainfluenzae* (Herbst & Snell 1948; 1949) and some strains of *Neisseria* (Martin, Pelczar & Hansen 1952). Later putrescine, spermidine and spermine were shown to be essential also for the growth of a mammalian cell line (Ham 1964).

As to the regulation of polyamine biosynthesis in mammalian tissue the important discovery of the putrescine forming enzyme, i.e. ornithine decarboxylase was made in 1968 in rat prostate gland, regenerating rat liver and chick embryo (Pegg & Williams-Ashman 1968; Jänne & Raina 1968; Russell & Snyder 1968). Ever since there have been extensive and intense investigations on the physiological significance of these amines which have been shown to affect a large number of cellular reactions, including

stabilization of membranes and subcellular fractions, involvement in virus multiplication, interaction with prokaryotic and eukaryotic DNA and RNA and participation in several stages of protein synthesis (for ref. see Cohen 1971; Bachrach 1973; Russell 1973; Tabor & Tabor 1976; Jänne, Pösö & Raina 1978).

The ornithine decarboxylase activity which was found elevated in the developing chick embryo (Russell & Snyder 1968) was correlated to the earlier observations (Raina 1963; Caldarera, Barbiroli & Moruzzi 1965) of changes in the diamine and polyamine content of this tissue. These changes were closely paralleled by changes in protein and nucleic acid concentrations (Caldarera et al. 1965; Moruzzi, Barbiroli & Caldarera 1968). Also in the fetal rat putrescine and polyamine content increased on a parallel with ornithine decarboxylase activity (Russell, Gregory, Medina & Snyder 1969; Snyder & Russell 1969) followed by an increased concentration of RNA (Russell & McVicker 1972a).

During the last decade a considerable amount of work has confirmed a correlation between the ability of various hormones to stimulate growth and differentiation and their ability to affect a change in diamine metabolism at an early stage of their action. Systems with such a response include the effects of ACTH on adrenal glands of hypophysectomized rats (Levine, Nicholson, Liddle & Orth 1973), epidermal growth factor on developing chick embryo epidermis (Stastny & Cohen 1969; 1970), LH on the ovaries of immature (Kaye, Icekson, Lamprecht, Gruss, Tsafiriri & Lindner 1973). or adult (Kobayashi, Kupelian & Maudsley 1971) rats, 17β -oestradiol on the chick oviduct (Cohen, O'Malley & Stastny 1970) or the uterus of ovariectomized (Cohen et al. 1970; Russell & Taylor 1971) or immature (Kaye, Icekson & Lindner 1971) rats and GH on rat liver (Jänne, Raina & Siimes 1968; Jänne & Raina 1969; Russell, Snyder & Medina 1970).

In contrast to the great number of investigations of histamine, putrescine, spermidine and spermine in various physiological connections, our knowledge of cadaverine in mammalian tissue is scarce. Cadaverine was, together with putrescine, early found to be excreted in the urine of patients with cysteinuria (Udránszky & Baumann 1889). This

excretion was believed to be due to an impaired absorption of the precursor amino acids ornithine and lysine, from the intestinal lumen, leading to an increased diamine formation by intestinal bacteria followed by absorption of the diamines and excretion by the kidneys (Milne, Asatoor, Edwards & Loughridge 1961). Cadaverine has also been described to be present in the urine of healthy humans (Perry & Schroeder 1963; Holder & Bremer 1966; Kohne & Bremer 1969) as well as in the urine of normal rats (Bremer & Kohne 1971), but still it was thought to be of intestinal origin. Evidence for biosynthesis of cadaverine from lysine in mammalian tissue cells was originally reported in the mouse kidney stimulated to growth by an anabolic steroid (Henningsson, Persson & Rosengren 1976).

In the present study the following items were investigated:

1. The urinary excretion of diamines and polyamines during rat pregnancy (I).
2. The influence of various ectomizing operations on the urinary excretion of diamines and polyamines in the pregnant rat (II).
3. The biosynthesis of cadaverine in the pregnant rat (III).
4. Changes in diamine and polyamine metabolism in rat ovary after administration of an exogenous gonadotrophin (IV).
5. The nature of the metabolic products formed from putrescine via the diamine oxidase pathway on incubating various tissues in vitro (V, VI).
6. The in vivo metabolism of radioactive putrescine in the pregnant rat (VI).
7. Changes in the contents of diamines and polyamines in tissues and body fluids of pregnant rats (II, III, VI).
8. The influence of the diamine oxidase inhibitor amino-guanidine on the metabolism of diamines and polyamines in rats during pregnancy and gonadotrophin administration (I-IV, VI).

METHODOLOGY

Experimental animals. Adult female rats (weight 220-340 g) and 28-day-old prepubertal female rats, of the Sprague-Dawley strain, were used. They were fed a diet of commercial rat chow or, when urine was to be collected, a semi-synthetic diet of standardized composition (Kahlson et al. 1958a). The amine content of the latter diet was: histamine 4, methylhistamine 0, putrescine 4, cadaverine 3, spermidine 2 and spermine 1 nmol/g (I). All rats had free access to tap water. The stage of the oestrus cycles of the adult rats was determined by microscopic examination of vaginal smears taken each morning and stained with methylene blue. Rats selected to become pregnant were allowed to mate in the pro-oestrus or early oestrus phase. They were then transferred to a male for 24 h. The next morning when mating was confirmed by the finding of spermatozoa in the vaginal smear and/or a vaginal plug was considered as the first day of pregnancy. No rat was allowed to suckle her young.

In some experiments adult female guinea-pigs (weight 830-950 g) and human placentae obtained at term were used (V).

Substances administered. Aminoguanidine hemi-sulphate, stored frozen until used, was injected in a dose of 10 mg/kg (I-IV, VI). Human chorionic gonadotrophin (1.5-200 IU) was freshly dissolved and administered in a volume of 0.1 ml (IV). 1,4-(¹⁴C)-Putrescine (2.5 μ Ci; 100 μ g) was stored frozen until given in a volume of 0.2 ml (VI). All substances were dissolved in 0.9 % NaCl and administered s.c.

Surgery. The rats, treated surgically under ether anaesthesia, were divided into five groups (II). Groups one to four consisted of pregnant rats, operated on the 19th day of pregnancy, while the rats of the fifth group were non-pregnant. The rats of the first group were subjected to sham-operations. A midline incision was made, through the skin and the abdominal muscles. The gravid uterine horn of one side was gently exposed and carefully examined. It was then put back into its proper place and the uterine horn of the

other side was examined. Then the abdominal wall was closed with silk sutures, first the muscular layer and then the skin.

In the pregnant rats of the second group the fetuses were removed while the placentae were left in place. For each fetus a small incision, parallel with the longitudinal axis of the uterine muscle fibers, was made on the anti-mesometrial side of the uterine wall. The fetus was gently pushed out, the umbilical cord cut and the incision closed with silk sutures.

In the third group of pregnant rats both fetuses and placentae were removed.

Rats of groups four and five were deprived of the ovaries and the uteri.

To promote postoperative recovery 4 ml 5.5 % (w/v) glucose was given at the awakening from the narcosis and later the same day 4 ml 0.9 % NaCl was injected s.c. The next day these injections were repeated and on the second day after the operations 4 ml saline was given.

Collection of urine. Urine was collected for determination of endogenous amounts of urinary diamines and polyamines (I, II, IV) and ^{14}C -putrescine and its radioactive metabolites excreted in the urine (VI).

On collecting urine the rats were kept in metabolism cages which provided facilities for collecting urine free from contamination by faeces. Hydrochloric acid, sufficient to give the urine a pH value below 2, was added to the collecting vessel to prevent bacterial formation and/or destruction of the amines during the 24 h collecting periods. The acidified urine sample was filtered through a Munktell's filter paper No. V20H and stored at -18°C until prepared for analysis of the relevant compounds.

Hydrolysis of urine. Hydrolysis was performed on urinary samples from rats before, during and after pregnancy (I). The samples were filtered through a $0.45\ \mu\text{m}$ Millipore filter. Ultrapure hydrochloric acid was added to 4 ml samples to a final concentration of 6 M. To remove dissolved air the samples were flushed with nitrogen. The tubes were then placed in an oven at 110°C for 16 h whereupon 1 mg EDTA

(ethylene diamine tetraacetic acid) per ml of urine was added and the samples evaporated to dryness at room temperature. The residue was resolved in 0.2 ml 30 % sulfosalicylic acid and 1.8 ml 0.20 M sodium citrate buffer (pH 2.2).

Preparation of body fluids and tissue samples for determination of diamine and polyamine content. To urinary samples (I, II, IV, VI) and to amniotic fluid (II) 30 mg sulfosalicylic acid and 1 mg EDTA were added per ml.

Blood, collected in a cooled beaker prepared with heparin and aminoguanidine, was withdrawn after cutting the carotid arteries (VI). To each ml blood 0.5 ml sulfosalicylic acid (60 mg sulfosalicylic acid and 0.6 mg EDTA/ml) was added and the samples were carefully pestled.

Tissues (about 200 mg) were rapidly removed and immediately homogenized in 3.5 ml sulfosalicylic acid (40 mg sulfosalicylic acid and 0.4 mg EDTA/ml).

Then the tissue samples were boiled in a water bath for 30 min. All samples were centrifuged at 800 x g for 15 min. The supernatant was adjusted to a pH value of 2.0 - 2.5 and filtered through a Millipore filter with a pore size of 0.22 μ m and stored at -18°C until analyzed for its content of diamines and polyamines.

Determination of non-isotopic diamines and polyamines and of 14 C-putrescine and its radioactive metabolites. The separation and quantitative estimation of the relevant compounds in the various samples were performed on a strongly acidic cation exchange resin (Durrum DC 6A) with a bed measuring 6 x 78 mm by means of an automatic amino acid analyzer as described by Henningsson (1978). The separation column was water jacketed and its temperature was kept at 60°C. For analysis of radioactive metabolites of 14 C-putrescine excreted in the urine or formed in incubates (V, VI) four buffers, pumped through the column at a flow-rate of 80 ml/h, were used. The buffers were of the following composition: Buffer A: 19.60 g tri-sodium citrate 2-hydrate and 10 ml 10 M hydrochloric acid/l, pH 3.50; Buffer B: 34.33 g tri-sodium citrate 2-hydrate/l, pH 6.30; Buffer C: 13.72 g tri-sodium citrate 2-hydrate, 45.58 g sodium chloride and 150 ml propanol/l, pH 6.20; Buffer D: 34.33 g

tri-sodium citrate 2-hydrate and 143.20 g sodium chloride/l, pH 5.90. The elution times for the four buffers were 44, 44, 48 and 40 min, respectively. The stream, after having passed the column, was split. One half of the effluent proceeded to a fraction collector and the other half could be used for coupling to ninhydrin. Aliquots of a total of 70 fractions were transferred to a liquid scintillation solution (Instagel, Packard) and the radioactivity was measured in a liquid scintillation spectrometer (Packard, TRI-CARB, 2002).

In determining endogenous amounts of diamines and polyamines in tissue samples and body fluids, in order to get a shorter elution time, only the last three buffers were used (I-IV, VI). These buffers were pumped through the column at a flow-rate of 60 ml/h for 20, 64 and 51 min, respectively. After passing the column the eluate was mixed with ninhydrin. The amines, having formed a coloured complex by reacting with ninhydrin in a bath of boiling water, then passed through a colorimeter. The absorption maximum of the coloured complex was at 570 nm. The amine content was quantified by measuring the peak areas of the curves representing the ninhydrin complex.

Preparation of tissue samples for analysis of enzymatic activities. The rats and guinea-pigs were killed by cervical dislocation and exsanguinated whereupon the appropriate tissues were rapidly removed, cooled on ice, minced with scissors and homogenized in a Dounce type of homogenizer (25 strokes with pestle) in cold 0.1 M sodium phosphate buffer (pH 6.9 or 7.2) containing 0.2 % (w/v) glucose (V) and 5×10^{-4} M dithiothreitol and 10^{-4} M EDTA (III, IV, VI). The minced tissue slices or the homogenate was used for determination of in vitro metabolism of putrescine. For determination of decarboxylase activities, i.e. formation of cadaverine and putrescine, the supernatant obtained at centrifugation at $20,000 \times g$ for 20 min at 4°C was used (III, IV).

Determination of cadaverine and putrescine formation. Formation of cadaverine and putrescine was determined by estimating the amount of $^{14}\text{CO}_2$ evolved by decarboxylation of

17 -

^{14}C -carboxyl-labelled DL-lysine and ^{14}C -carboxyl-labelled DL-ornithine, respectively (III, IV).

Aliquots of the 20,000 x g supernatant were incubated in a reaction mixture (final volume 1.0 ml) containing 10^{-2} M ^{14}C -carboxyl-labelled DL-lysine monohydrochloride (sp.act. 10 or 100 $\mu\text{Ci}/\text{mmol}$) and 10^{-3} M pyridoxal-5-phosphate or 10^{-4} M ^{14}C -carboxyl-labelled DL-ornithine monohydrochloride (sp.act. 500 $\mu\text{Ci}/\text{mmol}$) and 10^{-5} M pyridoxal-5-phosphate. The incubations were carried out with agitation in a water-bath at 37°C for 60 and 30 min, respectively. The reactions were terminated by the gentle tipping of 0.7 ml 2 M citric acid into the incubate from a side arm of the incubation vessel. Blanks were provided by incubating acidified samples. To obtain low blank values in the test for cadaverine formation the ^{14}C -lysine solution was flushed with a gas mixture of 4 % CO_2 and 96 % N_2 for 30 min before use. The $^{14}\text{CO}_2$ expelled during the incubation was trapped on a 10 x 25 mm piece of Munktell's filter paper No. 005 prepared with 100 μl of hydroxide of Hyamin 10-X (1 M solution in methanol). Maximum absorption of the carbon dioxide formed during the incubation was achieved by continued mechanical shaking for another 45 min. The filter paper was then transferred to a liquid scintillation solution (Bray 1960) and the radioactivity was measured.

For stoichiometric determinations of cadaverine formation supernatants were incubated in the presence of aminoguanidine hemi-sulphate (final conc. 2×10^{-5} M) (III). The amount of cadaverine formed in the incubate was measured by the automated amino acid analyzer and compared to the amount of $^{14}\text{CO}_2$ expelled during the incubation.

Determination of in vitro metabolism of ^{14}C -putrescine.

The in vitro metabolism of putrescine was studied by estimating the amount of ^{14}C -metabolites formed from ^{14}C -putrescine during the incubation with various tissues (V, VI).

Minced tissue or tissue homogenate was incubated at 37°C in a reaction mixture with a final volume of 1.5 ml, containing 10^{-4} M 1,4-(^{14}C)-putrescine monohydrochloride (sp.act. 0.125 or 1.25 mCi/mmol) and 10^{-4} M pyridoxal-5-phosphate. Blanks were provided by preincubation with

2×10^{-5} M aminoguanidine hemi-sulphate. The incubation was stopped by the tipping of 0.7 ml 2 M hydrochloric acid or 0.7 ml 12.6 % sulfosalicylic acid into the incubate.

$^{14}\text{CO}_2$, formed during the incubation, was trapped as described above. Chromatographic separation of other ^{14}C -metabolites and ^{14}C -putrescine was performed using the automatic amino acid analyzer (V, VI). The following ^{14}C -metabolites were determined: GABA, Δ^1 -pyrroline and some unidentified compound(s). The identity of GABA was confirmed by rechromatography on the column using a buffer system described by Hamilton (1962) for separation of amino acids (VI).

The amount of Δ^1 -pyrroline was also estimated by the method devised by Okuyama & Kobayashi (1961) (V). Aliquots of the incubates were mixed with saturated NaHCO_3 and the pH adjusted to a value of 9.4-9.6. Toluene was added to the samples and the ^{14}C - Δ^1 -pyrroline formed in the incubates was extracted into this organic solvent by vigorous shaking for 4 min. Next, the samples were centrifuged at $900 \times g$ for 2 min and aliquots of the toluene phase transferred to a liquid scintillation solution (Permablend, Packard) and the radioactivity was measured.

Radioactive putrescine, GABA and the unidentified compound(s) were also determined by thin layer electrophoresis (V). Aliquots of the incubate were centrifuged at $80 \times g$ for 20 min. A standard carrier solution containing unlabelled putrescine and GABA was added to the supernatant. The samples were mixed with absolute ethanol to precipitate the proteins and stored at -18°C until electrophoresis was performed. Next, the mixture was centrifuged, the supernatant evaporated to dryness, the residue resolved in 70 % ethanol and applied to a thin-layer plate (TLC-plates silica gel 60, Merck). The thin-layer electrophoresis was performed at 20 V/cm for 70 min and about 3°C using a pyridine-acetate buffer (pH 4.8) (Fischer & Bohn 1957). The relevant fractions were removed in zones and the silica gel powder was transferred to vials containing scintillation solution (Permablend, Packard) for determination of the radioactivity.

Determination of $^{14}\text{CO}_2$ in the expired air. The expiration

of $^{14}\text{CO}_2$ was determined after the injection of ^{14}C -putrescine to rats (VI). During a five h period (10 x 30 min) immediately after the injection of ^{14}C -putrescine the rat was placed in a cylindrical plexi-glass metabolism chamber with a volume of 700 ml which was swept with a stream of air at a constant flow-rate of 600 ml/min. Recordings of expired $^{14}\text{CO}_2$ from pregnant rats treated with aminoguanidine were made for 5 x 30 min. The air stream was directed to a series of vials containing 5 ml of hydroxide of Hyamin 10-X (1 M solution in methanol) for absorption of the expired $^{14}\text{CO}_2$. Aliquots of the hydroxide were transferred to a liquid scintillation solution (Bray 1960) and the radioactivity was determined. Correction was made for loss of methanol during the collecting periods.

Statistics. The values presented are mostly the arithmetic means and the S.E. of mean. For statistical evaluation of the data Student's t-test for paired or unpaired observations was used. The 0.05 level of probability was accepted as significant.

RESULTS AND COMMENTS

URINARY EXCRETION OF DIAMINES AND POLYAMINES IN THE PREGNANT RAT

Urinary excretion of diamines and polyamines in undisturbed pregnancy. The urinary excretion of histamine, methylhistamine, putrescine, cadaverine, spermidine and spermine was studied in rats before, during and after undisturbed pregnancy (I). The relation between the urinary content of the different amines during the course of pregnancy in one of the rats is shown in Fig. 1A.

The daily fluctuations in the urinary excretion of histamine before and during the first two weeks of pregnancy were not particularly great, neither among the different rats nor in the individual animals. On the 15th day of pregnancy the histamine excretion began to rise and then increased continuously to a maximum value 8-17 times the basal level 2-4 days before parturition. At parturition the urinary histamine excretion fell steeply and immediately after the birth of the young the basal level was obtained. Thus, the findings of Kahlson et al. (1958a) were confirmed. In the present study the urinary content of methylhistamine, an important metabolite in histamine degradation, was also determined. The urinary excretion of methylhistamine during pregnancy closely followed that of histamine. The maximum increase of methylhistamine excretion, estimated at 3-9 times the basal level, might however be falsely too low as an irrelevant peak interfered with the methylhistamine peak in chromatograms of urine samples with low levels of methylhistamine.

The pattern of urinary excretion of the diamine putrescine during pregnancy was similar to that of histamine and methylhistamine, i.e. no noteworthy change during the first two weeks of pregnancy and a highly elevated excretion during the last trimester. Peak values, 5-7 times the basal level were noted 3-4 days before parturition. After that the urinary putrescine excretion fell towards the basal level which after the birth of the young was rapidly resumed.

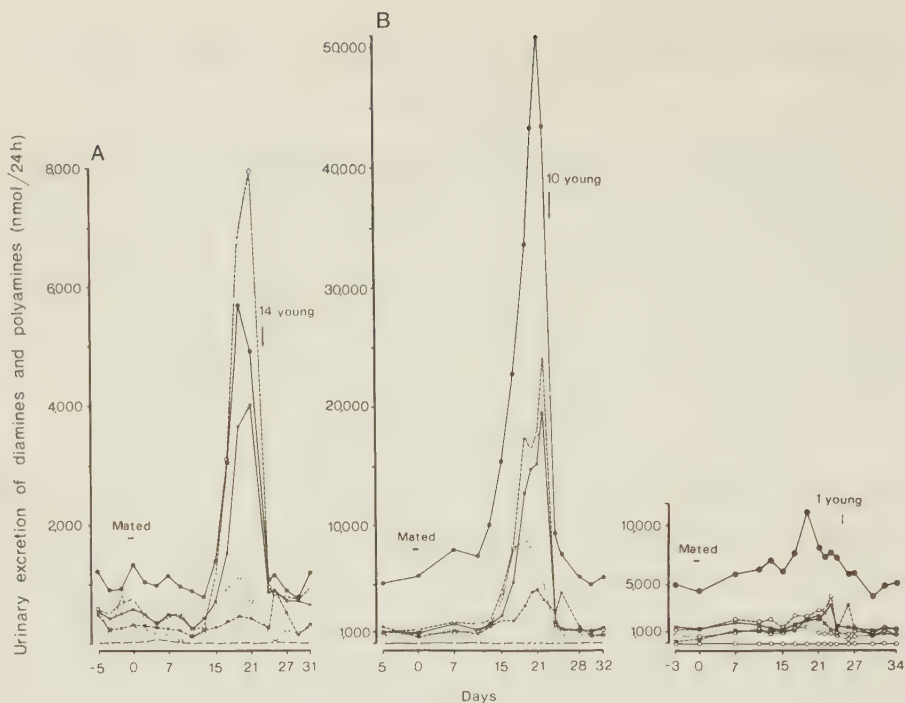


Fig. 1. Urinary excretion (nmol/24 h) of histamine (o--o), methylhistamine (x--x), putrescine (●--●), cadaverine (o....o), spermidine (x--x) and spermine (o—o) in rats before, during and after pregnancy. A: Undisturbed pregnancy in a rat giving birth to 14 young on the 23rd day of pregnancy. B: rats under the influence of aminoguanidine; one rat gave birth to 10 young on the 24th day of pregnancy and the other was delivered of one young by Caesarian section on the 25th day of pregnancy.

The urinary excretion of cadaverine was lower than that of the other diamines. On the 15th day of pregnancy a distinct increase in the excretion of cadaverine was found and on the 19th to 20th day peak values, 4-10 times the basal level, were obtained.

In the pregnant rat the urinary excretion of spermidine displayed a biphasic course with one peak before parturition and another occurring after delivery. The maximum urinary excretion during undisturbed pregnancy occurred after the birth of the young (Fig. 1A). On the 17th day of pregnancy the spermidine excretion rose and attained its

first peak 2-4 times the basal excretion 2-4 days before parturition. Thereafter the spermidine excretion declined towards the basal level until 1-2 days after the birth of the young when the second peak, about twice the first peak in magnitude, appeared. At the end of the period studied, i.e. about ten days after parturition the basal level of urinary spermidine excretion was again resumed.

The urinary excretion of spermine was much lower than that of any of the other amines studied and no change in its excretion was obtained during the course of pregnancy.

Urinary excretion of diamines and polyamines in pregnant rats treated with aminoguanidine. In view of the profound elevations in diamine oxidase activity in plasma and various tissues during pregnancy in the rat, the urinary excretion of the diamines and polyamines was studied under the influence of the diamine oxidase inhibitor aminoguanidine (I).

Fig. 1B depicts the results obtained in two of the rats, where the one to the left gave birth to 10 young and the one to the right was delivered of one young by Caesarian section.

It is obvious that essentially the same pattern of excretion of diamines and polyamines occurred in the pregnant rats treated with aminoguanidine though the basal levels as well as the peak values were highly elevated as compared to the values obtained in undisturbed pregnancy (Fig. 1). Thus, during the last trimester a distinct and steep increase in the excretion of histamine, methylhistamine, putrescine, cadaverine and spermidine ensued with peak values a few days before the birth of the young. Of the peak values noted, those for putrescine were the highest under the influence of aminoguanidine. Noteworthy were also the highly elevated peak values of cadaverine and methylhistamine. Regarding spermidine excretion a second peak appeared a few days after delivery as during undisturbed pregnancy though during aminoguanidine treatment the two peaks were almost equal in size.

Administration of aminoguanidine did not affect the

urinary excretion of spermine, neither before, during nor after pregnancy.

In the two rats in Fig. 1B that gave birth to 10 and one young, respectively there was a considerable difference in the elevation of the urinary output of diamines and polyamines during the last third of pregnancy. Thus, in the urine of the mother that had only one young the increase in the amine excretion was rather small. It has been shown that the increase in histamine excretion is larger the greater the number of young in the litter (Kahlson et al. 1958a). The present results agree with this assumption and suggest that the same may be valid also for other amines. A reason for the impaired amine excretion in the rat having only one young might however be some obstetrical complication as the young had to be delivered by Caesarian section. Thus, our information on this point has to be extended.

Urinary excretion of diamines and polyamines in rats subjected to various ectomizing operations. The findings of a highly elevated urinary diamine and polyamine excretion in rat pregnancy raised the question of the origin of these amines. Therefore the influence of various ectomizing operations, performed on the 19th day of pregnancy, on the urinary excretion of diamines and polyamines was investigated in rats with, as well as without daily injections of aminoguanidine (II). The urinary amine content was determined in samples from the 7th day of pregnancy, the day before surgery and for three days postoperatively. Following the various operations essentially the same pattern of urinary excretion of diamines and polyamines was seen whether the rats had received aminoguanidine or not. The elevation in the excretion of spermidine was however more pronounced in the pregnant rats treated with aminoguanidine, making these rats more suitable for evaluating the effects of the ectomizing operations. The results obtained from the aminoguanidine treated rats are shown in Fig. 2.

Kahlson et al. (1958b) showed that fetectomy abolished the increased urinary excretion of histamine in the

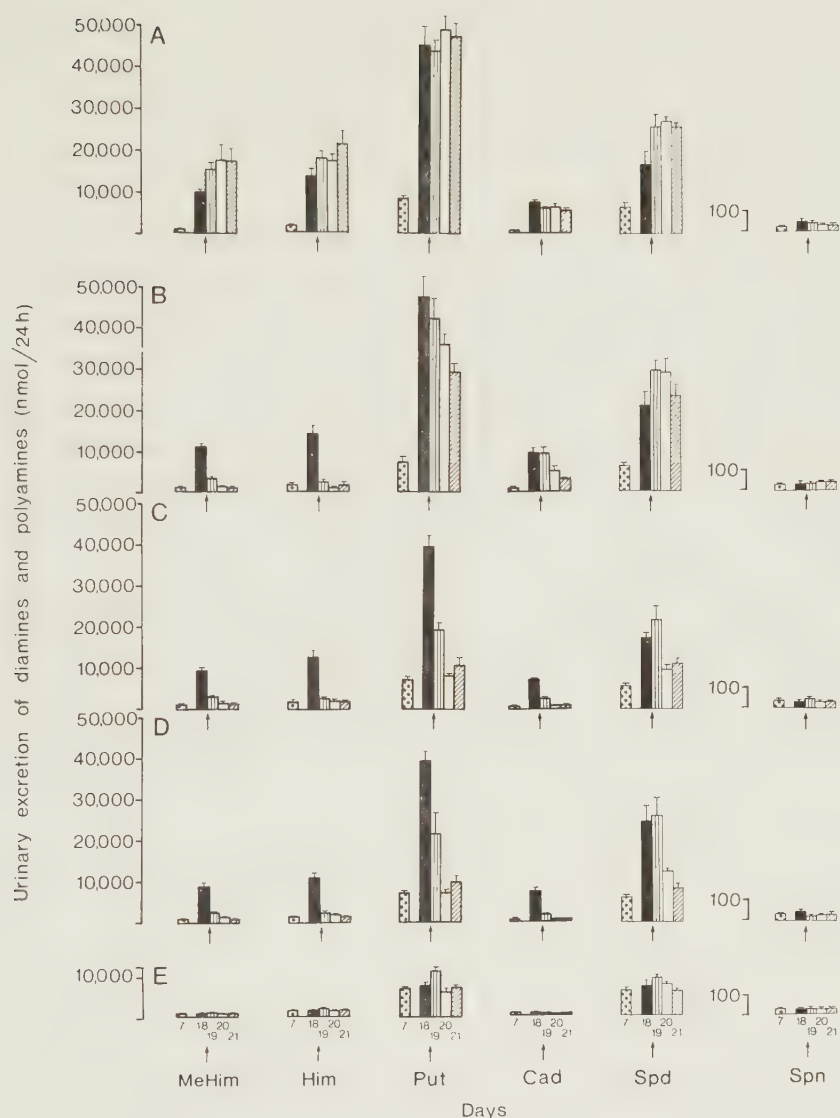


Fig. 2. Urinary excretion (nmol/24 h) of methylhistamine (MeHim), histamine (Him), putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spn) in aminoguanidine treated rats subjected to various ectomizing operations. A-D: pregnant rats, E: non-pregnant rats. The arrow denotes the time of surgery. A: sham-operation, B: fetectomy, C: fetectomy + placentectomy, D: removal of gravid uterus + ovaries, E: hysterectomy + ovariectomy. Each column represents the mean value $\pm$ S.E. of mean of determinations from four rats.

pregnant rat. This finding was confirmed in the present study and shown also to apply to methylhistamine as removal of the fetuses reduced the excretion of histamine as well as methylhistamine towards the level of non-pregnant rats (Fig. 2B). No further change in the urinary excretion of histamine and methylhistamine was attained by removing also the placentae and/or the uterus and the ovaries (Figs. 2C and 2D).

Removal of the fetuses on the 19th day of pregnancy did not abolish the increased urinary excretion of putrescine and cadaverine in the pregnant rat (Fig. 2B) while removal of also the placentae significantly reduced the urinary excretion of these diamines (Fig. 2C). The combined hysterectomy and ovariectomy of the pregnant rats did not further change the urinary excretion of putrescine while it caused some further depression in the urinary content of cadaverine (Fig. 2D).

Neither spermidine was depressed to the non-pregnant level by fetectomy (Fig. 2B). Removal of also the placentae and/or the uterus and the ovaries did not alter the spermidine excretion during the first day postoperatively but caused a significant reduction on the second and third day after surgery (Figs. 2C and 2D).

There were no significant changes in the urinary spermine excretion following any of the ectomizing operations (Fig. 2).

The combined hysterectomy and ovariectomy of the non-pregnant rats did not cause any significant changes in the urinary output of diamines and polyamines (Fig. 2E), indicating that the contribution of the uterus and the ovaries to the amount of diamines and polyamines excreted in the urine of the non-pregnant rat is negligible.

The effect of acid hydrolysis on urinary diamine and polyamine levels. To reveal if there were any significant amounts of diamines and polyamines excreted in conjugated form in the rat urine hydrolysis was performed on samples from untreated as well as aminoguanidine treated rats before, during and after pregnancy (I). Acid hydrolysis doubled the amounts of spermidine and spermine and elevated

the values of histamine and putrescine by about 20 %. The contents of methylhistamine and cadaverine were not increased after hydrolysis. Consequently, hydrolysis changed only the quantity but not the general pattern of excretion of any of the amines studied.

FORMATION OF CADAVERINE IN THE PREGNANT RAT

As to the origin of the increased amount of cadaverine excreted in the urine during the last third of rat pregnancy the ability of various tissues to form cadaverine in vitro was investigated on the 19th day of pregnancy when the urinary cadaverine excretion had been shown to be at its peak level (III).

Of the tissues examined (ovary, uterus, kidney, liver, fetus and placenta) the placenta was found to be most potent in the ability to form cadaverine. Further, it was shown that the fetal part of the placenta was responsible for the major part of the cadaverine forming capacity, in fact the synthesis of cadaverine in the maternal part was only 15 % of that in the fetal one.

The ovary of the pregnant rat also formed cadaverine at a high and, in comparison with the non-pregnant rat, significantly increased rate.

Parallel assays of $^{14}\text{CO}_2$ -production and the formation of cadaverine from ^{14}C -carboxyl-labelled lysine were performed to ensure that the evolution of $^{14}\text{CO}_2$ provided a valid measure of the biosynthesis of cadaverine. It was apparent that there was a fair accordance between the values obtained by the two methods showing that spurious release of $^{14}\text{CO}_2$ did not occur.

DIAMINE AND POLYAMINE METABOLISM IN RAT OVARY AFTER ADMINISTRATION OF HUMAN CHORIONIC GONADOTROPHIN

As the significantly increased formation of cadaverine in the ovary of the pregnant rat (III) might be caused by the hormonal status prevailing during pregnancy, the effect of human chorionic gonadotrophin (hCG) on diamine and polyamine metabolism in the rat ovary was investigated (IV).

Parallel determinations of cadaverine and putrescine formation in the gonadotrophin stimulated ovary were made. In addition the content of cadaverine, putrescine, spermidine and spermine in the ovary following administration of hCG was investigated. Determinations of the effect of hCG on the content of diamines and polyamines in the ovary and the urine of rats treated with aminoguanidine were also made.

Results concerning the in vitro formation of cadaverine and putrescine in the ovaries at various intervals after the administration of 50 IU of hCG are shown in Fig. 3. Already two h after the hormone injection the formation of cadaverine and putrescine was significantly increased. Peak values of the formation of both diamines were obtained 5 h after the hormone injection. The maximum formation of cadaverine and putrescine was 15 and 160 times the controls, respectively. At 12 h the formation of both cadaverine and putrescine was considerably lower, but had not reached the control values.

The formation of cadaverine and putrescine in the ovary was also determined 5 h after the injection of hCG in the dose-range of 1.5-200 IU. Maximum diamine formation was observed by doses between 25 and 50 IU. Doses above 50 IU were less effective.

The content of cadaverine, putrescine, spermidine and spermine was determined in the ovaries at 1-12 h after the injection of 50 IU hCG. The administration of hCG increased the content of the diamines, while there was a decrease in the level of the polyamines. The maximum increase in putrescine concentration, 10 times the control level, was reached 5-6 h after the hormone injection. The concentration of cadaverine in control ovaries was not

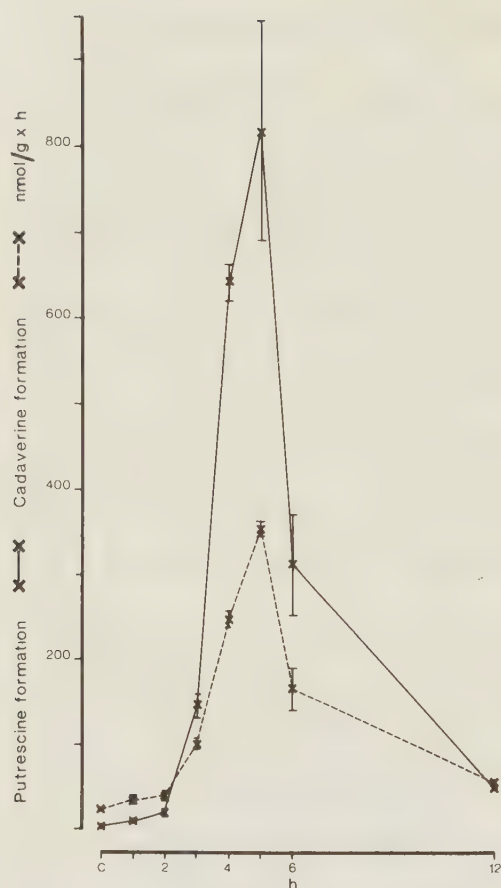


Fig. 3. Formation of cadaverine and putrescine (nmol/g x h) in the ovaries of rats injected with hCG (50 IU) at 28 days of age. The rats were sacrificed at different times after the injection. Control rats (C) received saline. Each point represents the mean value $\pm$ S.E. of mean. Controls: n=7, hCG-treated: n=4.

measurable but it increased on hormone administration and reached a maximum concentration after 5 h. The decrease in the level of the polyamines displayed different courses. While there was a fairly gradual decrease in the spermine content with a maximum reduction of 44 % 12 h after the hormone administration, the reduction of the spermidine concentration was about 15 % 2-5 h after the hormone administration and the control level was resumed and even slightly enhanced after 12 h.

Treatment of the rats with aminoguanidine elevated the content of cadaverine in the ovary, while the putrescine and polyamine content remained essentially unchanged in both control and hCG treated rats.

In the urine collected for 24 h the excretion of sper-

midine was significantly increased, while there was a slight but not significant increase in the urinary excretion of cadaverine, putrescine and spermine in the hormone treated animals.

DETERMINATION OF TISSUE DIAMINE OXIDASE ACTIVITY

When putrescine is incubated in the presence of purified diamine oxidase γ -aminobutyraldehyde is formed which is non-enzymatically transformed into the cyclic compound Δ^1 -pyrroline. In vitro measurements of diamine oxidase activity have been based on the determination of ^{14}C - Δ^1 -pyrroline formed from ^{14}C -putrescine. As in vivo experiments had shown a further metabolism of γ -aminobutyraldehyde to GABA and carbon dioxide it appeared relevant to examine whether these metabolites were formed also in vitro from ^{14}C -putrescine. If so, determination of only Δ^1 -pyrroline could not constitute a valid measure of tissue diamine oxidase activity. Therefore liver of guinea-pigs and human placental tissue obtained at term (V) as well as several tissues of pregnant and non-pregnant rats (VI) were incubated with ^{14}C -putrescine and the nature of the ^{14}C -metabolites formed in the incubates was determined.

In initial experiments when crude homogenate of gui-

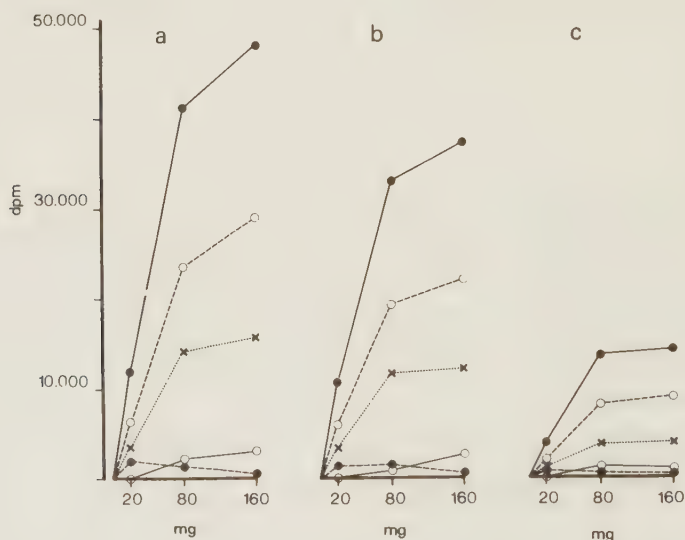


Fig. 4. Rate of formation of different metabolites from ^{14}C -putrescine in guinea-pig liver as related to the amount of tissue. The metabolites were CO_2 (o-o), Δ^1 -pyrroline (●-●), GABA (o-o) and some unknown compound(s) (x-x). The total amount of these metabolites is also given (●-●); a-c denote different animals.

nea-pig liver was used, in addition to ^{14}C - Δ^1 -pyrroline, large amounts of $^{14}\text{CO}_2$ were formed while at incubation with a 20,000 x g supernatant of the liver homogenate only Δ^1 -pyrroline was formed. The catabolism of putrescine was considerably higher in the homogenate than in the supernatant. On investigating the incubates for other metabolites of ^{14}C -putrescine GABA and some unknown compound(s) were found to be the major metabolites formed via the diamine oxidase pathway.

Experiments were performed to study the relationship between the rate of formation of different putrescine metabolites and the amount of tissue in the incubate (Fig. 4). A striking finding was that the tiny amounts of Δ^1 -pyrroline formed varied inversely with the amount of tissue incubated. Similar results were obtained when the time of incubation was varied (V).

Also in human placenta radioactive carbon dioxide, Δ^1 -pyrroline, GABA and some unknown compound(s) were formed on incubation with ^{14}C -putrescine. The metabolite formed in the largest quantity was Δ^1 -pyrroline, indicating a low aldehydedehydrogenase activity in the placental tissue (V).

The results obtained on incubating different tissues of pregnant and non-pregnant rats with ^{14}C -putrescine are shown in Fig. 5. Again, formation of the mentioned metabolites, derived via the diamine oxidase pathway, was recorded. In the pregnant rat the highest diamine oxidase activity per unit of weight was found in the maternal part of the placenta, followed by the uterus and the distal ileum. The main metabolite formed from putrescine by these tissues was Δ^1 -pyrroline, in fact it constituted about 94 % of the total amount of metabolites formed. In the other tissues of the pregnant rat examined, the pattern of metabolites was different. In the ovary the formation of Δ^1 -pyrroline and GABA was equal in magnitude, while in the fetal placenta and the liver GABA was the metabolite found in the highest concentration constituting an average of 57 % of the total amount of metabolites formed. In the kidney the diamine oxidase activity was low; only small

amounts of GABA and carbon dioxide were formed.

In the non-pregnant rat all the tissues examined showed a low diamine oxidase activity; the only exception was the ileum where the diamine oxidase activity was of the same magnitude as in the pregnant rat (Fig. 5).

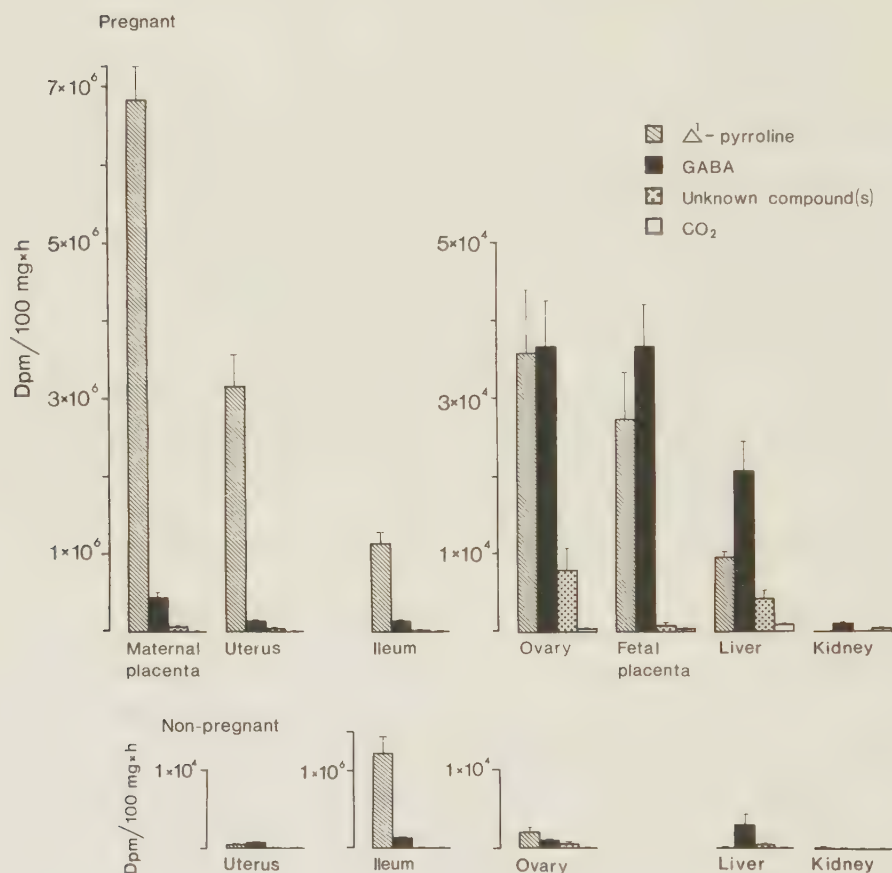


Fig. 5. Rate of in vitro formation of various metabolites from ^{14}C -putrescine in tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of gestation. Each column is the mean value $\pm$ S.E. of mean of 4 determinations.

To test the reliability of the methods, the putrescine metabolites formed (with the exception of $^{14}\text{CO}_2$) and the amount of radioactive putrescine remaining after the incubation were assayed in different systems (V, VI). In general a good agreement was obtained by the different methods used.

IN VIVO METABOLISM OF PUTRESCINE IN THE PREGNANT RAT

1,4-(^{14}C)-Putrescine (2.5 μCi ; 100 μg) was injected to untreated rats before pregnancy, on the 19th day of pregnancy and ten days after parturition (VI). Immediately after the injection of the isotope radiorespirometric recordings of the expired $^{14}\text{CO}_2$ were made for 10 x 30 min in one group of rats and urine was collected for 3 x 24 h for analysis of its radioactivity in another group of rats.

In Fig. 6 the excretion of $^{14}\text{CO}_2$ following the injection of ^{14}C -putrescine is shown. The putrescine was rapidly metabolized; within 30 min of the administration of the isotope 9 % of the injected radioactivity was recovered as

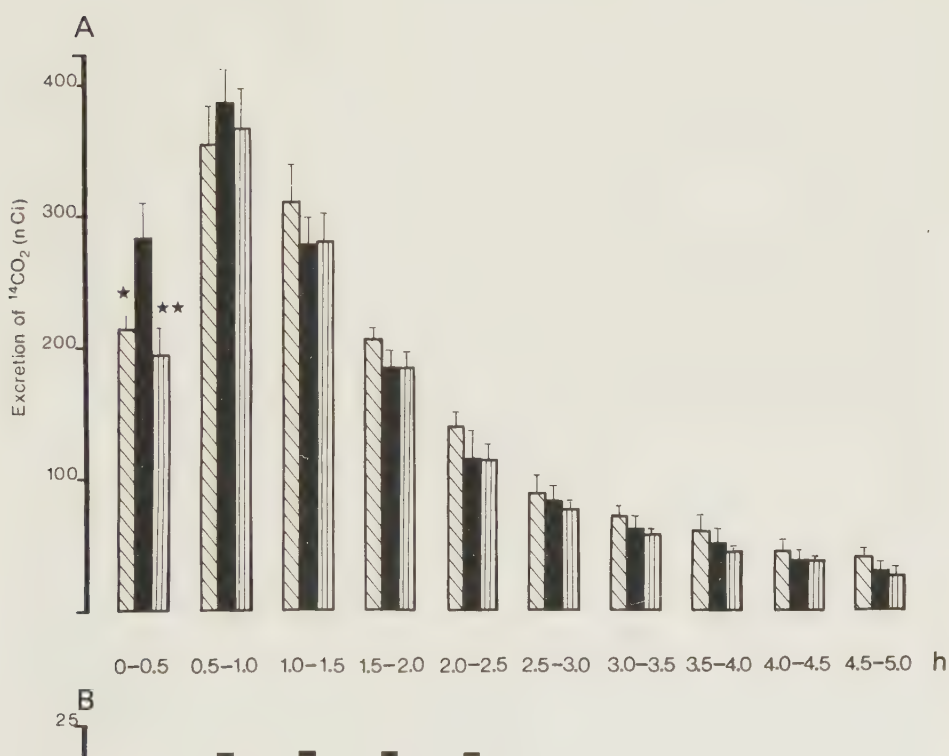


Fig. 6. Excretion of $^{14}\text{CO}_2$ (nCi) after the injection of ^{14}C -putrescine (2.5 μCi ; 100 μg) to rats. $\square$: rats before pregnancy, $\blacksquare$: rats on the 19th day of pregnancy, $\square$: rats 10 days after delivery. A: untreated rats, B: rats treated with aminoguanidine. The columns are mean values $\pm$ S.E. of mean. Untreated rats: n=4, rats treated with aminoguanidine: n=2. * = $p < 0.05$, ** = $p < 0.01$ versus pregnant rats.

$^{14}\text{CO}_2$ in the rats examined before and after pregnancy. During pregnancy this fraction was significantly increased, constituting 12 % of the injected radioactivity. However, during the whole period of 5 h there was no significant increase in the $^{14}\text{CO}_2$ excretion of the pregnant rats. Forty-eight per cent of the given radioactivity was expired within 2.5 h and 60 % within the whole recording period of 5 h. As seen in Fig. 6B administration of aminoguanidine almost completely inhibited the excretion of $^{14}\text{CO}_2$. In the pregnant rats treated with the inhibitor 0.8 % of the injected radioactivity was recovered as $^{14}\text{CO}_2$ during the 2.5 h recording period.

In the urine of the pregnant rats, collected for three days following the administration of ^{14}C -putrescine, 16 % of the injected radioactivity was recovered. Corresponding figures for radioactivity in the urine of the rats before and after pregnancy were 19 % and 22 %, respectively. The major part of the radioactivity excreted in the urine was found during the first day following the administration of ^{14}C -putrescine. In rats not given aminoguanidine GABA, the unidentified compound(s) and unmetabolized putrescine were excreted in the urine collected for 24 h following the ^{14}C -putrescine administration. GABA and the unidentified compound(s) were excreted in almost equal amounts by the rats both before, during and after pregnancy while ^{14}C -putrescine was excreted in significantly less amounts by the pregnant rats. Under the influence of aminoguanidine the pregnant rats excreted 8 times more unmetabolized putrescine in the urine, while the excretion of the unidentified compound(s) and GABA was significantly decreased. In addition, in the urine of the aminoguanidine-treated pregnant rats, small amounts of ^{14}C -spermidine appeared; no urinary ^{14}C -polyamines were found in the urine of the untreated rats.

CONTENT OF DIAMINES AND POLYAMINES IN BODY FLUIDS AND TISSUES OF THE PREGNANT RAT

Amniotic fluid. Amniotic fluid was collected from untreated and aminoguanidine-treated rats on the 19th day of pregnancy (II). Methylhistamine was not found in measurable amounts while histamine, cadaverine, putrescine, spermidine and spermine were found in equal amounts in both groups of rats indicating either a low diamine oxidase activity in the amniotic fluid or an inability for aminoguanidine to pass into it.

Blood. Determination of the content of cadaverine (unpublished results), putrescine and polyamines was made in blood collected from untreated and aminoguanidine-treated rats on the 19th day of pregnancy as well as from non-pregnant aminoguanidine-treated rats (VI). Cadaverine, putrescine and spermidine were elevated in the blood of aminoguanidine-treated pregnant rats as compared to the non-pregnant ones, while there were no evident changes in the concentration of spermine.

Tissues. The content of cadaverine (III), putrescine, spermidine and spermine (VI) was determined in tissues from rats corresponding to those from which blood was collected. In the following tissues the diamine and/or polyamine content was determined: ovary, uterus, kidney, liver, ileum, fetus and placenta.

In the untreated pregnant rat the highest concentration of cadaverine and putrescine was observed in the ovary and the placenta. Aminoguanidine treatment of the pregnant animals generally elevated the cadaverine content of the tissues investigated (III). An elevation in the content of putrescine on aminoguanidine treatment was seen in the uterus, kidney and ileum (VI), while the content of the polyamines in the various organs of the pregnant rat did not obviously change under the influence of aminoguanidine (VI).

Notable were the significant increases in the content of cadaverine, putrescine and spermidine in the ovaries of the pregnant aminoguanidine-treated rats as compared to the non-pregnant ones.

DISCUSSION

A relation between diamine metabolism and reproduction in the female rat was, as mentioned in the Introduction, first indicated by the discovery of large increases in histaminase activity in placental tissue and plasma during pregnancy. This connection became stronger by the finding that the concentration of histamine gradually increased in the developing rat fetus (Misrahy 1946) concomitant with a high fetal formation of histamine and an elevated histamine excretion in the urine of the pregnant rat during the last trimester (Kahlson et al. 1958a; Angervall et al. 1960; Kahlson et al. 1960; Telford & West 1961; Burkhalter et al. 1962). The highly elevated urinary excretion of histamine in the pregnant rat was confirmed in the present investigation (I, II). In addition to histamine, the urinary excretion of methylhistamine, a quantitatively important metabolite in histamine degradation, was studied (I, II). The methods for determination of this metabolite have been complicated and time consuming (White 1966) and the knowledge of the excretion of methylhistamine during pregnancy has been fragmentary. However, our liquid chromatographic method (Henningsson 1978) made it possible to determine methylhistamine as well as other diamines and polyamines in one and the same analysis of a sample.

Thus, it was shown that during the last part of rat pregnancy the elevated urinary histamine excretion was accompanied by a corresponding increase in methylhistamine excretion (I, II). Further it was shown that the presence of the fetuses but not the placentae was essential for the increased urinary output of both histamine and methylhistamine as removal of the fetuses with the placentae left intact abolished not only the increased urinary histamine excretion but also that of methylhistamine (II). This indicates that the fetuses might be capable not only to form large amounts of histamine but also to methylate a large portion of the newly formed amine as suggested by Lindberg Lindell & Westling (1963b). However, the possibility of methylation by maternal tissues must also be considered.

In the present study which was broadened to comprise other biogenic amines, namely the diamines putrescine and cadaverine and the polyamines spermidine and spermine, a striking elevation in the urinary diamine and polyamine excretion during the last trimester of rat pregnancy was shown (I, II). These findings further strengthen the idea of a relation between changes in diamine metabolism and the hormonal state of pregnancy. In this connection it should be mentioned that injections of 17β -oestradiol to immature or ovariectomized rats result in a rapid stimulation of the putrescine forming enzyme in the uterus (Cohen et al. 1970; Kaye et al. 1971; Russel & Taylor 1971) followed by increased concentrations of uterine putrescine and polyamines (Russel & Taylor 1971). Endogenous oestrogens obviously cause similar alterations in uterine diamine and polyamine metabolism; a distinct elevation of ornithine decarboxylase activity has been found on the pro-oestrus day in the rat uterus and elevated polyamine levels have been noted at pro-oestrus and oestrus (Hernández, Ballesteros, Mendez & Rosado 1973). Likewise, during early pregnancy in rat, coinciding well in time with the ovarian oestrogen surge, an increased ornithine decarboxylase activity has been found in the uterus (Saunderson & Heald 1974; Heald 1976).

While removal of the fetuses without dislodging the placentae abolished the increased excretion of histamine and methylhistamine in the urine of the pregnant rat, the increased urinary excretion of putrescine, cadaverine and spermidine was not much affected, indicating a crucial importance of the presence of the placentae but not the fetuses for the elevated output of these amines (II).

As removal of the placentae changes the hormonal state prevailing during pregnancy the reduction of the urinary excretion of putrescine, cadaverine and spermidine following removal of both fetuses and placentae does not necessarily indicate that these amines are of placental origin. However, the site of cadaverine formation in the pregnant rat was investigated by a more direct approach (III). Of the various tissues examined the fetal placentae displayed the highest in vitro formation of cadaverine. This finding suggests that the elevated urinary excretion of cadaverine

during pregnancy may be a consequence of the high rate of cadaverine formation in the fetal placenta. The same may be valid for putrescine as the placenta has been shown to possess a high capacity of forming also this diamine (Gutha & Jänne 1976a; Maudsley & Kobayashi 1977).

In the rat not allowed to suckle her young the urinary excretion of spermidine displayed a biphasic course with one peak before parturition and another occurring after delivery (I). In the lactating rat preliminary results indicate the occurrence of a prolonged and augmented urinary output of spermidine. In the blood of rats during pregnancy and lactation the spermidine level has been found to be elevated (Lundgren & Oka 1978). Also the content of spermidine in the mammary gland was found strikingly high during these conditions (Neish & Kay 1968; Russel & McVicker 1972b). These findings make it possible that the mammary gland might be one source of the elevated amount of spermidine found in the urine of the rat in connection with pregnancy.

Also in human pregnancy there are elevations in the urinary excretion of diamines and polyamines (Bjurö et al. 1961; Russell, Levy, Schimpff & Hawk 1971; Fujita, Nagatsu, Maruta, Ito, Senba & Miki 1976; Russell, Giles, Christian & Campbell 1978).

A considerable number of observations has shown a correlation between enhanced synthesis of putrescine and polyamines and rapid cell growth or cell proliferation (for ref. see Tabor & Tabor 1976; Jänne et al. 1978). Until recently nothing had been disclosed on the formation of cadaverine in mammalian tissues (Henningsson et al. 1976). As mentioned, in the pregnant rat, the fetal placenta showed a high ability to form cadaverine (III). In addition, in the ovary of the pregnant rat a significantly elevated formation of cadaverine was found (III), an observation which led to experiments where the exposure of rats to an exogenous gonadotrophin was studied (IV). It was shown that the ovary stimulated by hCG, like the ovary of the pregnant rat, formed cadaverine at a highly increased rate (IV). The elevated diamine formation was con-

sistent with an increased amine content in the ovary of the pregnant as well as the gonadotrophin stimulated rat.

Regarding putrescine, a conspicuous stimulation of the formation of this diamine has been found in the ovary in the evening of pro-oestrus, i.e. between the surge of LH and ovulation, suggesting an association between putrescine metabolism and the ovulatory process (Kobayashi et al. 1971). The present study where the formation of both putrescine and cadaverine was studied following hCG administration suggests that cadaverine metabolism might also be involved in this process. The use of specific inhibitors of the appropriate enzymes involved in the biosynthesis of diamines and polyamines could be useful tools in investigating a possible influence of inhibited amine formation on ovarian function. Preliminary experiments suggest that α -difluoromethyl ornithine, an irreversible inhibitor of ornithine decarboxylase (Metcalf, Bey, Danzin, Jung, Casara & Vever 1978), might interfere with ovarian function in rats.

Aminoguanidine, which because of its potency (Schuler 1952) frequently has been used as an inhibitor of diamine oxidase, doubled the concentration of cadaverine in the ovary of the pregnant rat (III). With respect to the finding of diamine oxidase activity in the ovary of the pregnant (VI) and gonadotrophin stimulated (Andersson, Henningsson & von Schéele 1980) rat, this enzyme may function as a regulator of the intra-ovary level of cadaverine.

Thus, in the ovary of the pregnant rat there are profound changes both in biosynthesis (Guha & Jänne 1976b; III) and degradation (VI) of diamines. The ovary was also the tissue that showed the largest changes in diamine (III, VI) and polyamine (VI) content in connection with pregnancy. Studies on biosynthesis of diamines and polyamines and on diamine oxidase activity in isolated ovarian compartments during pregnancy and the reproductive cycle would be of great interest to get a better insight into the possible function of diamine and polyamine metabolism in ovarian physiology.

The present results showing a regular pattern of urinary cadaverine excretion during pregnancy (I) and a high

formation of cadaverine in the placenta and the ovary of the pregnant (III) and gonadotrophin stimulated (IV) rat, indicate that cadaverine can be formed by mammalian tissue cells, and is then not a product of bacterial decarboxylation of lysine. This belief is strengthened by the observation of a cadaverine formation in the mouse kidney stimulated to growth by an anabolic steroid (Henningsson et al. 1976) and the occurrence of cadaverine in the blood and brain of germ-free mice (Stepita-Klauco & Dolezalova 1974). The present results also support the view of a connection between diamine metabolism and the hormonal status prevailing in pregnancy and are other examples of elevated diamine formation in relation to growth.

As to the physiological significance of cadaverine it should be noted that in microorganisms cadaverine has been suggested to possess some of the biological activity of putrescine and spermidine, possibly by the formation of a spermidine analog (Dion & Cohen 1972). A recent report describes a conspicuous formation of cadaverine and aminopropylcadaverine in cultured Ehrlich ascites carcinoma cells after depletion of putrescine and polyamines by inhibiting ornithine decarboxylase with α -difluoromethyl ornithine (Alhonen-Hongisto & Jänne 1980).

Thus, parallel studies of the metabolism of cadaverine and putrescine would benefit a better understanding of diamine metabolism not only in reproductive physiology but also in other physiological connections.

The employment of α -difluoromethyl ornithine in murine pregnancy (Fozard, Grove, Part & Prakash 1979) has provided strong evidence that changes in diamine metabolism are essential for the continuation of normal mammalian embryogenesis. Administration of α -difluoromethyl ornithine to mice during pregnancy resulted in a greatly retarded fetal development followed by resorption and/or expulsion of the fetuses. The period when administration of α -difluoromethyl ornithine displayed contragestational effects corresponded exactly to a period of large rises in uterine ornithine decarboxylase activity and amine concentrations. The effect of α -difluoromethyl ornithine was opposed by aminoguanidine (Fozard, Grove, Part & Prakash 1980).

In rat pregnancy high levels of diamine oxidase have been found in the plasma and various other tissues (Zeller 1940a; Roberts & Robson 1953; Kobayashi 1964; Kim & Harris 1973; VI). None the less, substantial amounts of diamines appeared unmetabolized in the urine in undisturbed pregnancy (Kahlson et al. 1958a; I). To obtain a more precise measure of the in vivo amine formation as reflected in the urinary excretion the break-down via the diamine oxidase pathway was inhibited by aminoguanidine. Under the influence of this inhibitor the excreted amounts, particularly of putrescine and cadaverine, were greatly increased (I, II) indicating that oxidative deamination via diamine oxidase is a major route of catabolism of these compounds in rats.

Thus, in the pregnant rat with its highly elevated diamine oxidase activity in plasma and several tissues, it was of interest to investigate the metabolic fate of injected radiolabelled putrescine. The in vivo metabolism of putrescine was investigated by measurement of the expiration of $^{14}\text{CO}_2$ and the excretion of ^{14}C -metabolites in the urine after the injection of ^{14}C -putrescine. The pregnant rat expired more $^{14}\text{CO}_2$ during the first half hour after ^{14}C -putrescine administration. Otherwise the amount of $^{14}\text{CO}_2$ expired and the amount of ^{14}C -metabolites excreted in the urine were remarkably similar in pregnant and non-pregnant rats. According to the present in vitro measurements of putrescine metabolism the main metabolite formed from ^{14}C -putrescine by the maternal placenta and the uterus was Δ^1 -pyrroline, indicating a low activity of aldehyde dehydrogenase in these tissues. Thus, the increased diamine oxidase activity in these tissues might not be paralleled by an increase in the enzymes catalyzing the further metabolism of putrescine, a fact that would contribute to the minor difference found in the in vivo metabolism of ^{14}C -putrescine of the pregnant and non-pregnant rats (VI). In the female rat there is an increased biosynthesis and accumulation of putrescine in the reproductive organs during pregnancy (Guha & Jänne 1976a; 1976b; Maudsley & Kobayashi 1977). Thus the slight difference in the metabolism of ^{14}C -putrescine might also be due to a greater dilution of

^{14}C -putrescine by unlabelled putrescine in the pregnant rat. Loading of rats with unlabelled putrescine has been shown to diminish the amount of $^{14}\text{CO}_2$ recovered during a 2 h period after the injection of ^{14}C -putrescine (Sourkes & Missala 1979). Also, the extremely rapid catabolism of ^{14}C -putrescine seen even in the non-pregnant rat probably rapidly decreases the substrate concentration of ^{14}C -putrescine. This might be an additional reason why increased expiration of $^{14}\text{CO}_2$ could be demonstrated only during the first half hour after the administration of the isotope.

The present study clearly demonstrates the importance of diamine oxidase for the in vivo metabolism of ^{14}C -putrescine in the rat. In the urine, after administration of ^{14}C -putrescine, GABA was found to be excreted. The other metabolite of putrescine shown to constitute the main urinary metabolite, was some unknown compound(s). Determination of the amount of polyamine excretion has been suggested as a biochemical test for the detection of early cancer (Russell et al. 1971). However, determination of endogenous amounts of the unidentified compound(s) found in our studies could possibly become a more sensitive tool in revealing early cancer as this compound(s) seems to be the main metabolite of ^{14}C -putrescine excreted in the urine. After inhibition of diamine oxidase with aminoguanidine the expiration of $^{14}\text{CO}_2$ was almost completely inhibited and the urinary excretion of GABA and the unidentified compound(s) was decreased while the amount of unmetabolized ^{14}C -putrescine was highly elevated. Only after administration of aminoguanidine ^{14}C -spermidine could be quantitated in the urine supporting the view of a preference for the metabolism of putrescine via diamine oxidase rather than conversion to polyamines.

The in vitro studies, using putrescine as a substrate for diamine oxidase, showed that mammalian tissues produce several products of ^{14}C -putrescine via the diamine oxidase pathway (V, VI). Further it was shown that the pattern of metabolites formed varied with the tissue investigated. While in guinea-pig liver Δ^1 -pyrroline constituted only a minor fraction of the total amount of metabolites formed (V) it was the major metabolite of ^{14}C -putrescine formed

by the maternal placenta, ileum and uterus of the pregnant rat (VI). Thus, Δ^1 -pyrroline alone can be used for determination of diamine oxidase activity in certain tissues but measurements of the total amount of metabolites formed from putrescine are necessary in other tissues to obtain a valid measure of the diamine oxidase activity (V, VI).

Most interesting was the finding of GABA as a major metabolite of putrescine formed by the guinea-pig liver and several tissues of the pregnant rat, suggesting a possible functional significance for GABA in these tissues. Notably, GABA has been found to stimulate protein synthesis (Tewari & Baxter 1969; Sandoval & Tapia 1975) and may thus have a function similar to that ascribed to the polyamines. Similarly, products of the oxidative deamination of cadaverine might be of importance in various cellular activities.

In most mammalian tissues putrescine has been considered mainly to serve as a precursor of the polyamines. Formation of polyamines from ^{14}C -putrescine was not detectable in the tissues investigated in the pregnant rat (VI) or in the ovaries of the immature rat after gonadotrophin stimulation (Andersson et al. 1980) indicating a preference for the metabolism of putrescine via oxidative deamination rather than conversion to polyamines. The biological significance of diamine oxidase is yet not settled but a better understanding of its metabolic function may provide a further insight not only into the physiological implication of this enzyme but also into that of diamines and polyamines in pregnancy as well as other biological conditions.

SUMMARY AND CONCLUSIONS

1. In the rat, during the last third of pregnancy, there was a greatly increased urinary excretion of histamine, methylhistamine, putrescine, cadaverine and spermidine. These findings strengthen the idea of a relation between changes in diamine and polyamine metabolism and the state of pregnancy. Administration of aminoguanidine further elevated the excreted amounts. The benefit of using aminoguanidine to obtain a more precise measure of the in vivo amine formation as reflected in the urinary excretion in the rat was emphasized.
2. Removal of the fetuses with the placentae left in place abolished the increased urinary excretion of histamine and methylhistamine while the excretion of cadaverine, putrescine and spermidine was still highly elevated. Removal of the fetuses and the placentae reduced the urinary excretion of also these amines to the level of non-pregnant rats. The additional excision of the uterus and the ovaries did not cause any additional effects except for the excretion of cadaverine which was further reduced. The observations indicate that the presence of the fetuses but not the placentae was essential for the elevated urinary output of methylhistamine and histamine while the presence of the placentae but not the fetuses was of crucial importance for the increased urinary amounts of cadaverine, putrescine and spermidine in the pregnant rat.
3. In the pregnant rat the highest in vitro formation of cadaverine was found in the fetal part of the placenta, followed by the ovary. These organs also exhibited the highest concentration of cadaverine. These experiments further support the view of the importance of the placenta for the increased amounts of cadaverine found in the urine of the pregnant rat. Administration of aminoguanidine generally increased the tissue content of cadaverine, indicating a role for diamine oxidase in regulating the endogenous amounts of this diamine.
4. Administration of human chorionic gonadotrophin increased the cadaverine and putrescine formation in the ovary of prepubertal rats. The elevated diamine formation was closely paralleled by an increased content of cadaverine and putrescine while the concentration of the polyamines was diminished. A role for diamine and polyamine metabolism in ovarian physiology is tentatively suggested.
5. The following radioactive metabolites, formed during in vitro incubation of ^{14}C -putrescine with guinea-pig liver, human placenta and various tissues of pregnant and non-pregnant rats, were found: Δ^1 -pyrroline, GABA, some unknown compound(s) and carbon dioxide. In guinea-pig liver the formation of Δ^1 -pyrroline, the amount of which was not related to the time of incubation or the amount of tissue used, constituted only a minor fraction of the total amount of metabolites formed. GABA was found to be a major metabolite of putrescine in guinea-pig liver and several tissues of the pregnant rat. It

is obvious that it is necessary to measure the total amount of metabolites formed in the incubate in order to obtain a valid measure of the tissue diamine oxidase activity. A possible functional significance of the products derived via oxidative deamination of diamines is suggested.

6. The injection of ^{14}C -putrescine to pregnant and non-pregnant rats was followed by the expiration of large amounts of $^{14}\text{CO}_2$. In the urine, in addition to unmetabolized putrescine some unknown compound(s) and GABA were excreted. Although a significantly increased expiration of $^{14}\text{CO}_2$ was registered during the first 30 min after the injection of ^{14}C -putrescine and a lower excretion of unmetabolized putrescine was found in the urine of the pregnant rats, the metabolism of ^{14}C -putrescine was remarkably similar in rats before, during and after pregnancy. Administration of aminoguanidine to pregnant rats almost completely inhibited the expiration of $^{14}\text{CO}_2$. In the urine increased amounts of unmetabolized putrescine were excreted while the excretion of GABA and the unidentified compound(s) was decreased. Small amounts of ^{14}C -spermidine were excreted in the urine of the aminoguanidine-treated rats. These experiments clearly demonstrated the importance of diamine oxidase for the in vivo metabolism of ^{14}C -putrescine in the rat; a preference for oxidative deamination via diamine oxidase rather than conversion to polyamines is suggested.
7. In amniotic fluid histamine, putrescine, cadaverine, spermidine and spermine were found in equal amounts whether the rats had received aminoguanidine or not. In the blood of the pregnant rat elevated amounts of cadaverine, putrescine and spermidine were found. In the ovaries of the pregnant rats significantly increased amounts of cadaverine, putrescine and spermidine were found while the concentration of spermine was decreased further suggesting a role for diamine and polyamine metabolism in ovarian physiology.

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APPENDIX: Publications I-VI.

INCREASED FORMATION OF DIAMINES AND POLYAMINES IN THE PREGNANT RAT

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SUMMARY

1. The urinary excretion of histamine, methylhistamine, putrescine, cadaverine, spermidine and spermine was examined before, during and after pregnancy in rats.

2. During the last third of undisturbed pregnancy a distinct and steep rise occurred in the excretion of all the amines studied except spermine. The peak values were found a few days before the birth of the young. In spermidine excretion a second peak was observed one or two days after delivery.

3. Before and during the first 2 weeks of gestation on a molar basis putrescine excretion was the greatest one. During the last trimester histamine was excreted in the largest amount.

4. Under the influence of the diamine oxidase inhibitor aminoguanidine the general pattern of excretion of diamines and polyamines in pregnant rats remained essentially unchanged but the total amount excreted increased. Most conspicuous was the great elevation of urinary contents of putrescine and cadaverine.

INTRODUCTION

A possible relation between diamine metabolism and pregnancy was first indicated by the discovery of elevated histaminase (diamine oxidase) activity in the human placental tissue (Danforth & Gorham, 1937) and in the serum of pregnant women (Marcou, Athanasiu-Vergu, Chiricéanu, Cosma, Gingold & Parhon, 1938). Large increases were later detected in the placental, uterine and serum histaminase activity of the pregnant rat (Roberts & Robson, 1953; Kobayashi, 1964).

A connexion between diamine metabolism and pregnancy became stronger after the discovery that the pregnant rat excreted large amounts of histamine in the urine during the last third of pregnancy. The increase in histamine excretion was found to be larger the greater the number of young in the litter, suggesting that the elevated histamine excretion was depending on the presence of the fetuses (Kahlson, Rosengren & Westling, 1958*a*). This view was supported by experiments showing that after the removal of the fetuses before term the increased urinary histamine excretion was rapidly abolished (Kahlson, Rosengren, Westling & White, 1958*b*).

A high rate of fetal histamine formation was shown also *in vitro*. The increased histamine formation of the fetus as reflected in the urinary excretion of the mother roughly corresponded to the period of rapid increase in weight of the fetuses

occurring after the 15th day of pregnancy. On the day of birth the histamine formation of the fetus was considerably lowered. This was reflected by a steep fall in urinary histamine excretion by the mother (Kahlson *et al.* 1958*b*).

The work related above, links histamine metabolism to a normal physiological process, i.e. pregnancy. During the past 10 years interest in some other biogenic amines, namely the diamine, putrescine, and the polyamines, spermidine and spermine, has greatly increased. Evidence has accumulated indicating that these amines are involved in processes associated with nucleic acid and protein synthesis (for references see Russell, 1973; Raina & Jänne, 1975). Another diamine, cadaverine, was found to be synthesized in increased amounts in the mouse kidney stimulated to growth by the administration of the anabolic steroid nandrolone (Henningsson, Persson & Rosengren, 1976).

The purpose of the present investigation was to examine if the urinary excretion of amines, other than histamine, was changed during rat pregnancy. In addition, the effect of aminoguanidine, a potent inhibitor of diamine oxidase (Schuler, 1952), on the pattern of urinary diamine and polyamine excretion during pregnancy was studied. A preliminary communication of the results has been given (Andersson, Henningsson & Rosengren, 1977).

METHODS

The observations were made on thirteen adult rats, weighing 230–290 g, of the Sprague-Dawley strain. They were fed a partly synthetic diet, 18–27 g (dry weight) daily and water *ad libitum*. The amine content of the diet was: histamine 4, methylhistamine 0, putrescine 4, cadaverine 3, spermidine 2 and spermine 1 n-mole/g. Before the actual experiment the rats were kept in metabolism cages every second day for at least 1 week. Their oestrus cycles were followed by examination of vaginal smears taken each morning and stained with methylene blue. Only rats with normal oestrus cycles were used. Rats chosen to become pregnant were allowed to mate in the pro-oestrus or early oestrus phase. They were transferred for 24 hr to a male kept on the same diet; mating was confirmed by the appearance of sperm in the vaginal smear and/or a vaginal plug. This was considered the first day of pregnancy. In two rats (nos. 8 and 10), the young were delivered by Caesarian section on the 25th and 24th day of pregnancy respectively. The other rats bore naturally on day 22–24 of pregnancy. No rat was allowed to suckle her young.

In a pilot experiment urine from a rat (no. 1) was collected in 24 hr samples every second day before, during and after undisturbed pregnancy and analysed for its amine content. Simultaneously urine was collected from two non-pregnant rats (nos. 2 and 3). In the other pregnant rats (nos. 4–6 and 8–11) the following urine samples were analysed for their amine content: one sample before mating, the samples of day 1, 7, 11, 13, 15 and 17 of pregnancy and from day 19 or 21 each 24 hr urine sample up to term. The amine content of urines from day 1, 2, 5, 7 and 9 after delivery was also determined. In addition, urine from three non-pregnant rats (nos. 7, 12 and 13) was assayed.

Of the rats used nos. 1–7 were studied under undisturbed conditions whereas rats nos. 8–13 were studied under the influence of the diamine oxidase inhibitor aminoguanidine. The inhibitor was injected subcutaneously (10 mg aminoguanidine hemi-sulphate/kg daily) for one week before the beginning of the urine collection and throughout the period studied.

On collecting urine the rats were kept individually in metabolism cages which provided facilities for collection of urine and faeces separately. Hydrochloric acid, sufficient to give the urine a pH value below 2, was added to the collecting vessel to prevent formation and/or destruction of the amines by bacteria during the 24 hr collecting periods. After rinsing of the collecting equipment with distilled water the volume of the sample was noted and the urine was filtered through a Munktell's filter paper no. V20H. The urine was then stored frozen until an aliquot was prepared for analysis of its diamine and polyamine content. For this preparation 1 mg EDTA (ethylene diamine tetraacetic acid) and 30 mg sulphosalicylic acid were added to

each millilitre of urine. The samples were then centrifuged at 800 *g* for 15 min whereafter the supernatant was adjusted to a pH value of 2.0–2.4 and filtered through a filter with a pore size of 0.22 μm (Millipore Corp.). The amine content of the filtrate was determined by liquid chromatography using an automatic amino acid analyser, LKB BIOCAL, 3201. We employed a strongly acidic cation-exchange resin (Durrum DC 6A) with a bed measuring 6 $\times$ 78 mm. An elution procedure, described in detail by Henningsson (1978) was employed. The composition and the elution times of the four buffers used were: buffer A: 0.20 M- Na^+ , pH 3.50, 44 min; buffer B: 0.35 M- Na^+ , pH 6.30, 44 min; buffer C: 0.90 M- Na^+ , pH 6.20, 48 min; buffer D: 2.80 M- Na^+ , pH 5.90, 40 min. In the latter part of this work, in order to get a shorter elution time only the last three buffers were used. These buffers were pumped through the column at a flow rate of 60 ml./hr for 20, 64 and 51 min respectively. The operating pressure for the actual column was 10–40 kP/cm² depending on which buffer was pumped through the column. The separation column was water-jacketed and its temperature was kept at 60 °C. After passing the column the eluate was mixed with ninhydrin (30 ml./hr). The amines, having formed a coloured complex by reacting with the ninhydrin in a bath of boiling water, then passed through a colorimeter. The absorption maximum of the coloured complex was at 570 nm. The amine content was quantified by measuring the peak areas of the curves representing the ninhydrin complex.

The accuracy of the method was within 3% and consecutive assays did not vary more than 1–2%. As to recovery, determined by ¹⁴C-labelled amines, it approached 100%. The sensitivity of the method, defined as the lowest reproducibly measurable amount of an amine, was about 1 n-mole.

In each pregnant rat hydrolysis of three urine samples (one before pregnancy, one during pregnancy and one post partum) was carried out. The samples were filtered through a filter with a pore size of 0.45 μm (Millipore Corp.). Ultrapure hydrochloric acid (Merck) was added to a final concentration of 6 M. To remove dissolved air the samples were flushed with nitrogen gas. This was done 5 times by reducing the pressure inside the hydrolysis tube to 15 mm Hg. The tubes were then placed in an oven at 110 °C for 16 hr, allowed to cool, 1 mg EDTA/ml. urine was added whereafter the samples were evaporated to dryness at room temperature. The residue was resolved in 0.2 ml. 30% sulphosalicylic acid and 1.8 ml. 0.20 M- Na citrate buffer, pH 2.2. Adjustment of pH and filtering through a 0.22 μm filter was then carried out and analysis was performed on the automatic amino acid analyser.

RESULTS

Urinary excretion of diamines and polyamines in non-pregnant rats and the influence of the diamine oxidase inhibitor aminoguanidine

The urinary excretion of histamine, methylhistamine, putrescine, cadaverine, spermidine and spermine was determined in five non-pregnant rats of which two were injected with the diamine oxidase inhibitor aminoguanidine. The results are seen in Table 1. In rats not given aminoguanidine the mean urinary excretion was: histamine 570, methylhistamine 360, putrescine 980, cadaverine 200, spermidine 230 and spermine 23 n-mole/24 hr. In rats receiving aminoguanidine the corresponding values were: histamine 1100, methylhistamine 1100, putrescine 5700, cadaverine 840, spermidine 770 and spermine 18. Thus, in these animals, aminoguanidine treatment elevated the excretion of putrescine by six times, whereas the excretion of cadaverine rose four times and that of histamine, methylhistamine and spermidine two to three times. As to spermine, no change in the excretion was noted.

Urinary excretion of diamines and polyamines in undisturbed pregnancy

The urinary excretion of the six amines studied was determined in four rats before, during and after undisturbed pregnancy. Each rat gave birth to a large litter (ten to fourteen young). The relation between the urinary contents of the different amines

TABLE 1. Average urinary excretion (n-mole/24 hr) of diamines and polyamines in non-pregnant rats (nos. 2, 3, 7, 12 and 13) based on day to day variations; number of observations in parenthesis. Rats nos. 12 and 13 were injected with aminoguanidine hemi-sulphate in a daily dose of 10 mg/kg. The figures are mean values $\pm$ s.e. of mean

Rat no.	Histamine	Methyl-histamine	Putrescine	Cadaverine	Spermidine	Spermine
2 (11)	440 $\pm$ 42.3	380 $\pm$ 37.4	920 $\pm$ 48.9	140 $\pm$ 23.8	220 $\pm$ 20.5	21 $\pm$ 2.40
3 (11)	460 $\pm$ 37.0	400 $\pm$ 32.6	1000 $\pm$ 79.9	260 $\pm$ 64.3	240 $\pm$ 29.3	23 $\pm$ 2.10
7 (4)	800 $\pm$ 61.5	310 $\pm$ 18.0	1000 $\pm$ 46.1	190 $\pm$ 47.3	240 $\pm$ 24.1	24 $\pm$ 5.53
12 (7)	1200 $\pm$ 58.0	1100 $\pm$ 71.4	5400 $\pm$ 363	800 $\pm$ 46.3	850 $\pm$ 132	16 $\pm$ 1.64
13 (6)	950 $\pm$ 60.8	1200 $\pm$ 88.6	6000 $\pm$ 627	880 $\pm$ 118	690 $\pm$ 89.5	19 $\pm$ 3.24

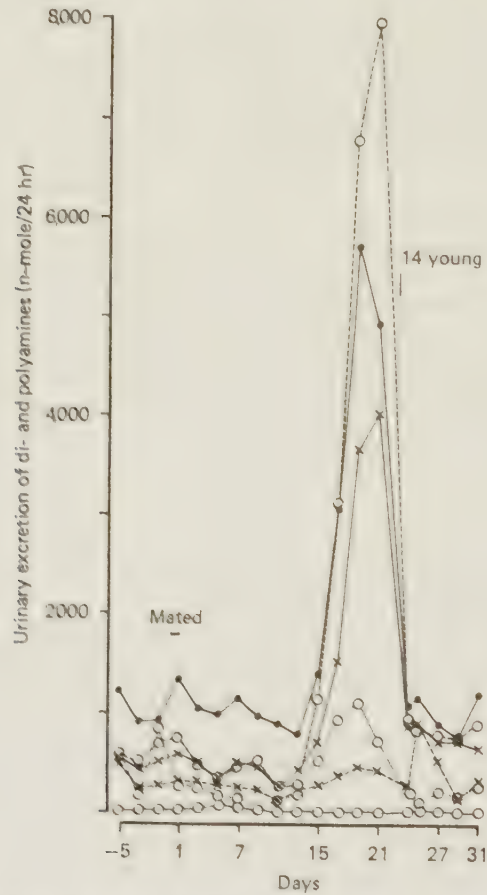


Fig. 1. Urinary excretion (n-mole/24 hr) of histamine (○---○), methylhistamine (×—×), putrescine (●—●), cadaverine (○...○), spermidine (×---×) and spermine (○—○) in rat no. 1 before, during and after undisturbed pregnancy. On the 23rd day of pregnancy fourteen young were born.

during the course of pregnancy in one of the rats is shown in Fig. 1. Results from the whole group of animals are given in Fig. 2 and Table 2.

Histamine and methylhistamine. As seen in Fig. 2 and Table 2 the daily fluctuations in histamine and methylhistamine excretion before and during the first 2 weeks of pregnancy were not particularly great, neither among the four rats nor in the

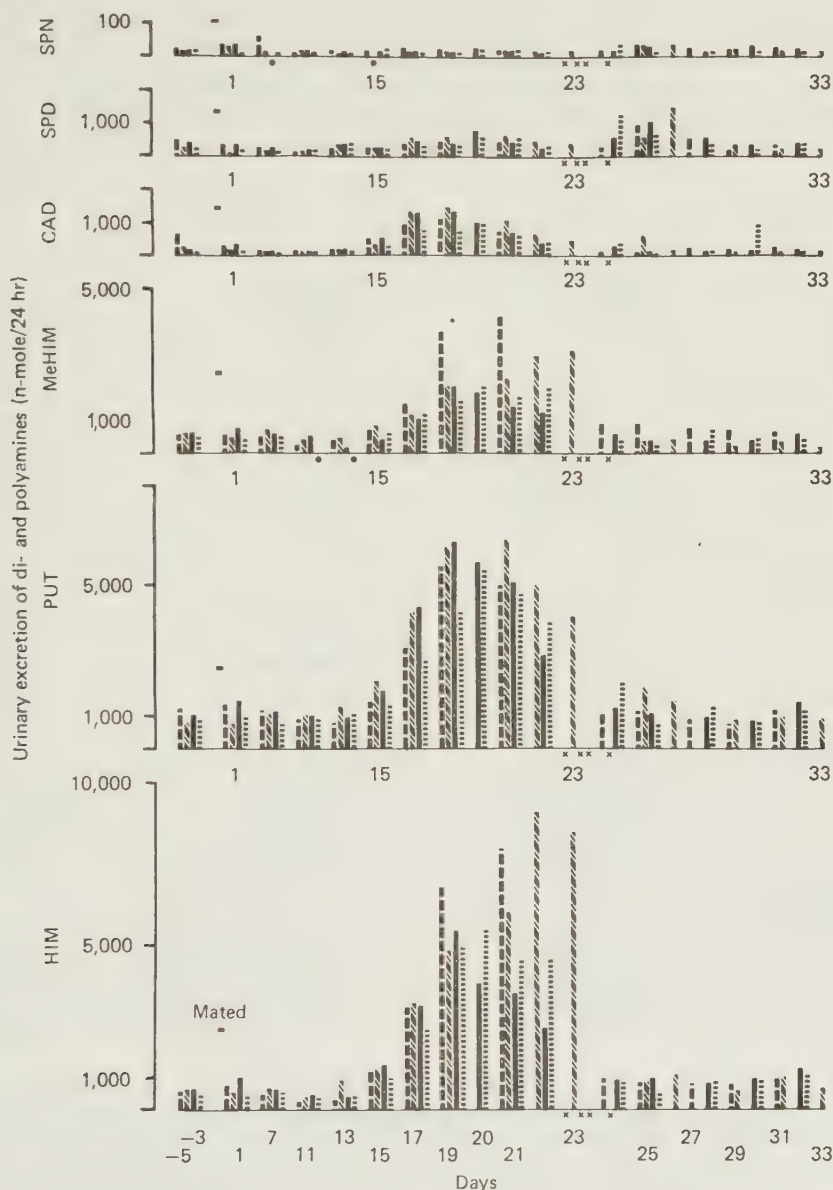


Fig. 2. Urinary excretion (n-mole/24 hr) of diamines and polyamines in four rats (nos. 1 (■), 4 (▨), 5 (▩) and 6 (▮)) before, during and after undisturbed pregnancy. Rat no. 4 gave birth to ten young on the 24th day of pregnancy, the others bore fourteen young on the 23rd day of pregnancy. x indicates the day of parturition. ● indicates sample lost.

individual animal. The mean value of histamine excretion before and up to the 11th day of pregnancy (basal level) varied between 450 and 680 n-mole/24 hr and that of methylhistamine between 470 and 610 n-mole/24 hr. On the 15th day of pregnancy the histamine excretion began to rise and then increased continuously to

TABLE 2. Urinary diamine and polyamine excretion (n-mole/24 hr) in four rats during undisturbed pregnancy. Rat no. 4 gave birth to ten young on the 24th day of pregnancy, the other three rats (nos. 1, 5 and 6) all gave birth to fourteen young on the 23rd day of pregnancy. I: basal level of amine excretion: mean values $\pm$ s.e. of mean of observations before and until day 11 of pregnancy. Number of observations in parentheses. II: peak value(s) of urinary amine excretion at the day of pregnancy indicated by the figures parentheses. III: ratio II/I.

Histamine	I	II	III
Rat no.			
1	510 $\pm$ 52.5 (9)	7900 (21)	15.5
4	550 $\pm$ 62.8 (4)	9000 (22)	16.5
5	680 $\pm$ 109 (4)	5400 (19)	8.1
6	450 $\pm$ 30.9 (4)	5500 (20)	12.3
Methylhistamine			
Rat no.			
1	470 $\pm$ 35.0 (9)	4200 (21)	9.0
4	540 $\pm$ 67.7 (4)	3100 (23)	5.8
5	610 $\pm$ 49.5 (4)	2000 (19)	3.3
6	470 $\pm$ 20.1 (3)	2100 (20)	4.4
Putrescine			
Rat no.			
1	1000 $\pm$ 52.4 (9)	5600 (19)	5.3
4	900 $\pm$ 71.4 (4)	6400 (21)	7.1
5	1200 $\pm$ 110 (4)	6300 (19)	5.5
6	850 $\pm$ 40.4 (4)	5400 (20)	6.4
Cadaverine			
Rat no.			
1	300 $\pm$ 88.0 (9)	1100 (19)	3.8
4	150 $\pm$ 32.8 (4)	1500 (19)	9.8
5	180 $\pm$ 46.4 (4)	1300 (19)	7.3
6	100 $\pm$ 13.4 (4)	950 (20)	9.4
Spermidine			
Rat no.			
1	290 $\pm$ 31.9 (9)	470 (19); 930 (25)	1.6; 3.2
4	160 $\pm$ 33.6 (4)	640 (21); 1500 (26)	4.1; 9.2
5	280 $\pm$ 44.3 (4)	770 (20); 1000 (25)	2.7; 3.6
6	180 $\pm$ 17.1 (4)	580 (21); 1200 (24)	3.2; 6.9

a maximum value 2-4 days before parturition. In the four rats the highest values observed were 8-17 times the basal level (Table 2). At parturition urinary histamine fell steeply and immediately after the birth of the young the basal level of urinary excretion was obtained. The pattern of urinary excretion of methylhistamine during pregnancy was similar to that of histamine and the maximum increase was estimated at 3-9 times the basal level. However, at the elution of the amines small amounts of an unknown substance partly overlapped the methylhistamine peak

making it difficult to exactly quantify the basal excretion of the amine. The amount of the unknown substance did not significantly change during pregnancy. Consequently the unknown compound interfered more with low concentrations of methylhistamine. For that reason the calculated basal level of methylhistamine in the urine may be falsely enhanced and the maximum increase during pregnancy even more than 3–9 times.

Putrescine. The pattern of urinary excretion of the diamine putrescine during pregnancy was similar to that of histamine and methylhistamine, i.e. no noteworthy change during the first two weeks and a highly elevated excretion during the last week of pregnancy. As seen in Table 2, the mean values for the four rats up to day 11 of pregnancy varied from 850 to 1200 n-mole/24 hr. On the 15th day of pregnancy (Fig. 2) an elevated output of putrescine occurred in each of the four rats. Peak values, varying from 5400 to 6400 n-mole/24 hr, were noted 3–4 days before parturition corresponding to a maximum excretion of 5–7 times the basal level. Thereafter the urinary putrescine excretion fell towards the basal level which after the birth of the young was rapidly resumed.

Cadaverine. As seen in Fig. 2 and Table 2 the basal urinary excretion of cadaverine was lower than that of the other diamines, varying from 100 to 300 n-mole/24 hr. On the 15th day of pregnancy a distinct increase in the excretion of cadaverine was found in all four rats. On the 17th day it was almost as high as on the 19th to 20th day when the peak values varying between 950 and 1500 n-mole/24 hr were observed.

Spermidine and spermine. In the pregnant rat the urinary excretion of spermidine displayed a biphasic course with one peak before parturition and another occurring after delivery. From Fig. 2 and Table 2 it can be seen that the maximum urinary excretion occurred not before but after the birth of the young. The mean urinary excretion before and during the first 2 weeks of pregnancy varied between 160 and 290 n-mole/24 hr in the four rats. On the 17th day of pregnancy the spermidine excretion rose and attained its first peak (470–770 n-mole/24 hr) 2–4 days before parturition. Thereafter the spermidine excretion declined towards the basal level until 1–2 days after the birth of the young when the second peak (930–1500 n-mole/24 hr) appeared. At the end of the period studied, i.e. about 10 days after parturition the basal level of urinary spermidine excretion was again resumed.

The urinary excretion of spermine was much lower than that of any of the other amines and no change in spermine excretion was observed during pregnancy (Fig. 2).

The effect of aminoguanidine on the excretion of diamines and polyamines in the pregnant rat

In non-pregnant rats, as shown above, aminoguanidine administration greatly elevated the urinary excretion of the diamines as well as that of spermidine. Further, as said in the Introduction, diamine oxidase activity of placenta, uterus and serum is elevated during pregnancy. Thus, it seemed of interest to study the influence of aminoguanidine on amine excretion in the pregnant rat. The results from two individual rats are summarized in Fig. 3 and those from the group of four in Fig. 4 and Table 3.

From the Figures it is obvious that essentially the same pattern of excretion of diamines and polyamines occurred in the pregnant rats whether they had received

aminoguanidine or not. During the last trimester of pregnancy a distinct and steep increase in the excretion of histamine, methylhistamine, putrescine, cadaverine and spermidine ensued with peak values a few days before the birth of the young. Of

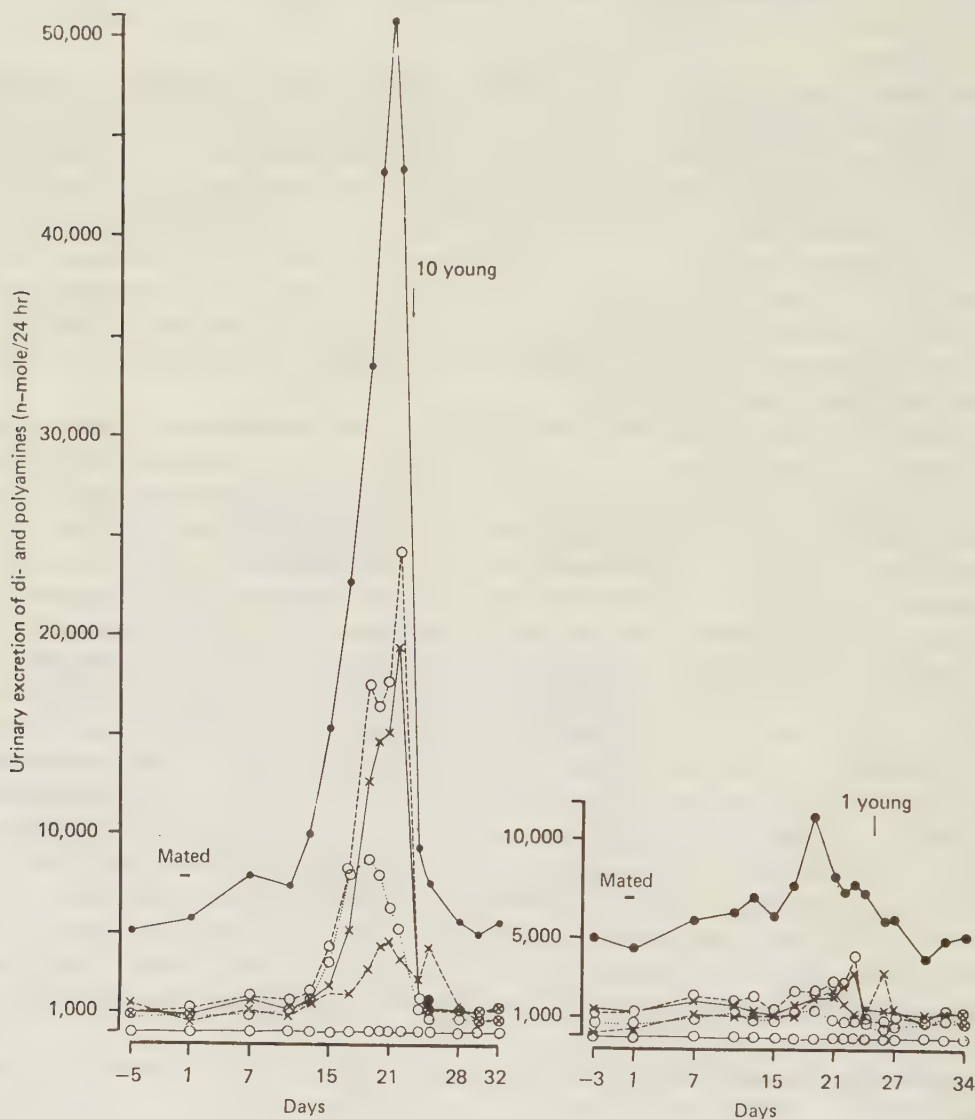


Fig. 3. Urinary excretion (n-mole/24 hr) of histamine ($\bigcirc$ --- $\bigcirc$), methylhistamine ($\times$ — $\times$), putrescine ($\bullet$ — $\bullet$), cadaverine ($\bigcirc$... $\bigcirc$), spermidine ($\times$ --- $\times$) and spermine ($\bigcirc$ — $\bigcirc$) in rat no. 11 (left) and rat no. 8 (right) before, during and after pregnancy. The rats were treated with aminoguanidine hemi-sulphate (10 mg/kg once daily). Rat no. 11 gave birth to ten young on the 24th day of pregnancy. Rat no. 8 was delivered of one young by Caesarian section on the 25th day of pregnancy.

the peak values noted, those for putrescine were the highest under the influence of aminoguanidine. Noteworthy is also the greatly elevated peak values of cadaverine and methylhistamine. Regarding spermidine excretion a second peak appeared a few

days after delivery as during undisturbed pregnancy although during aminoguanidine treatment the two peaks were almost equal in magnitude. In spermine excretion no change was noted during aminoguanidine administration in the pregnant rat.

Of the two rats represented in Fig. 3 one bore one young and the other bore ten young. The increase in the amine excretion was rather small in the rat which bore just one young. It has been suggested that the increase in histamine excretion is larger the greater the number of young in the litter (Kahlson *et al.* 1958*a*). Thus, the present results agree with this assumption; this may in addition hold for the other amines although our information on this point has to be extended.

The effect of acid hydrolysis on urinary diamine and polyamine levels

It may be argued that the amounts of free diamines and polyamines are inadequate measures of the total amounts of the urinary amines since they may in part be excreted in conjugated form. To test this possibility the urinary contents of the amines were examined after hydrolysis of the urine. Hydrolysis was performed on urinary samples from each of the eight rats before pregnancy, on the 19th day of pregnancy and 1–2 days after parturition. Hydrolysis doubled the amounts of spermidine and spermine whereas the values of histamine and putrescine were elevated by about 20 %. The increase after hydrolysis was the same in samples before, during and after pregnancy both of the untreated rats and of the rats treated with aminoguanidine. The contents of methylhistamine and cadaverine were not increased after hydrolysis. Consequently, the general pattern of the excretion of all the amines studied was found to be the same whether the urines were hydrolysed or not.

DISCUSSION

The observation that changes in formation of diamines *in vitro* are followed by corresponding changes in the urinary excretion of the amines or their metabolites offers possibilities to study *in vivo* changes in their metabolism (Kahlson *et al.* 1958*b*; Henningsson & Rosengren, 1975; Henningsson *et al.* 1976). Earlier studies have shown that in the female rat the normal rate of formation and mobilization of endogenous histamine can be followed continuously from day to day by observing the urinary excretion of the amine (for references see Kahlson & Rosengren, 1968). Amongst others it was shown that the rate of histamine formation in the rat fetus was high. This was reflected in an elevation of urinary histamine excretion on the 15th day of pregnancy. As the fetuses grew the histamine excretion climbed to a peak in the last 1–2 days before term when a precipitous fall in the rate of formation ensued.

Results have now been obtained suggesting a close parallelism between histamine biosynthesis and metabolism and that of putrescine, cadaverine and spermidine in rat pregnancy. Regarding putrescine it has been shown that in rat ovary the formation increases sharply on the 11th day of pregnancy and remains elevated almost until term (Guha & Jänne, 1976). In addition, a high putrescine formation has been found in the developing rat placenta (Maudsley & Kobayashi, 1977). These observations based on *in vitro* studies are in accordance with the results obtained in the present study on endogenous amine excretion which support the view that in the

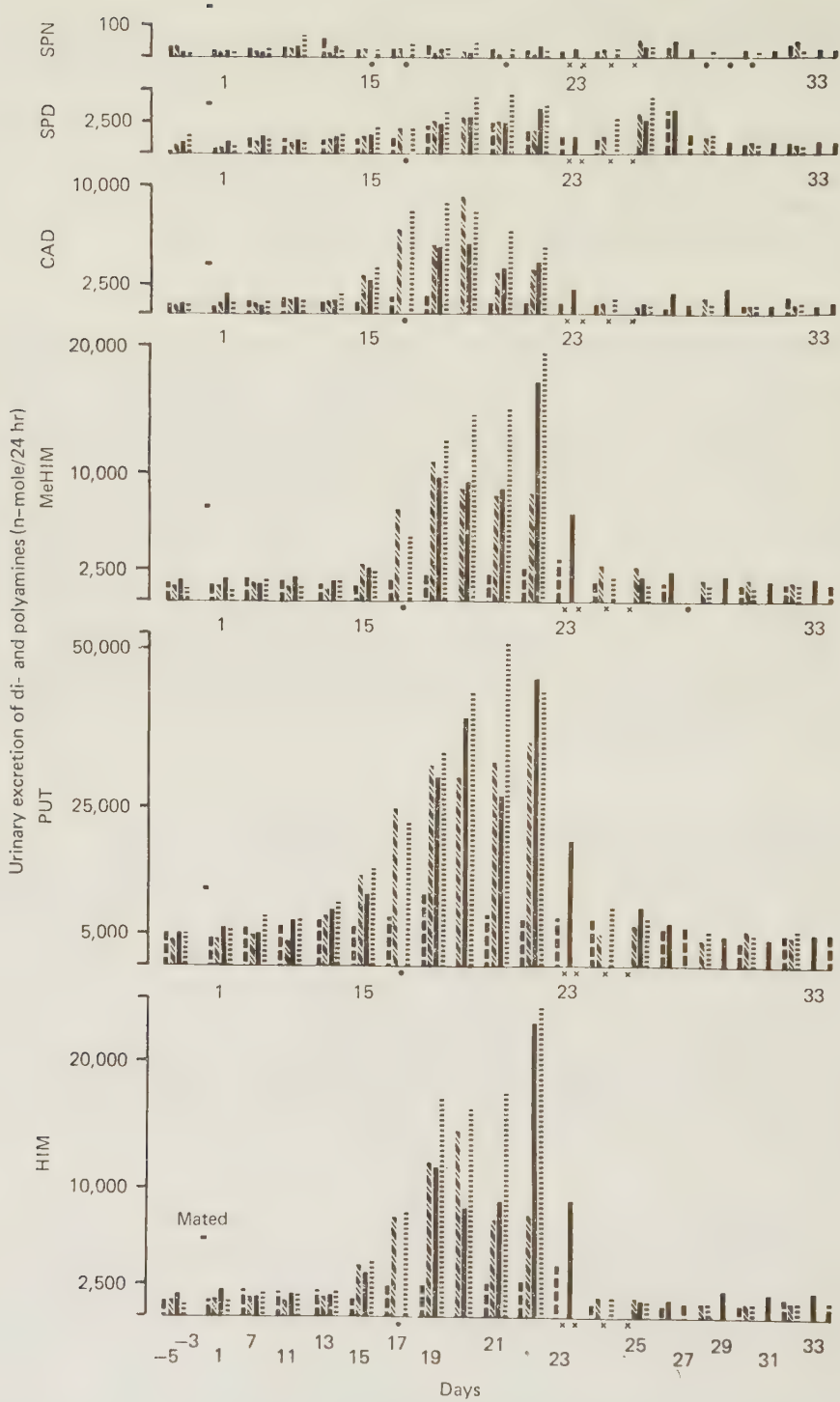


Fig. 4. For legend see opposite page.

TABLE 3. Urinary diamine and polyamine excretion (n-mole/24 hr) during pregnancy in four rats treated with aminoguanidine hemi-sulphate in a daily dose of 10 mg/kg. Rat no. 8 and rat no. 10 were delivered of one and thirteen young by Caesarian section on the 25th and 24th day of pregnancy, respectively. Rat no. 9 gave birth to thirteen young on the 23rd day of pregnancy and rat no. 11 gave birth to ten young on the 24th day of pregnancy. I: basal level of amine excretion: mean values $\pm$ S.E. of mean of observations before and until day 11 of pregnancy. Number of observations in parentheses. II: peak value(s) of urinary amine excretion at the day of pregnancy indicated by the figures in parentheses. III: ratio II/I.

Histamine	I	II	III
Rat no.			
8	1600 $\pm$ 231 (4)	4100 (23)	2.5
9	1400 $\pm$ 70.7 (4)	15000 (20)	10.6
10	1800 $\pm$ 135 (4)	23000 (22)	12.7
11	1400 $\pm$ 200 (4)	24000 (22)	16.9
Methylhistamine			
Rat no.			
8	1600 $\pm$ 126 (4)	3300 (23)	2.1
9	1300 $\pm$ 66.9 (4)	11000 (19)	8.5
10	1700 $\pm$ 96.3 (4)	17000 (22)	10.3
11	1200 $\pm$ 172 (4)	19000 (22)	16.1
Putrescine			
Rat no.			
8	5400 $\pm$ 432 (4)	11000 (19)	2.1
9	4300 $\pm$ 211 (4)	35000 (22)	8.1
10	6000 $\pm$ 510 (4)	45000 (22)	7.6
11	6600 $\pm$ 660 (4)	51000 (21)	7.8
Cadaverine			
Rat no.			
8	920 $\pm$ 140 (4)	1400 (19)	1.5
9	880 $\pm$ 102 (4)	7700 (20)	8.8
10	1100 $\pm$ 207 (4)	5400 (20)	4.9
11	910 $\pm$ 67.8 (4)	8700 (19)	9.6
Spermidine			
Rat no.			
8	740 $\pm$ 238 (4)	2400 (21); 3400 (26)	3.3; 4.6
9	730 $\pm$ 102 (4)	2800 (20); 3100 (25)	3.9; 4.2
10	1100 $\pm$ 79.9 (4)	3500 (22); 3400 (26)	3.3; 3.2
11	1000 $\pm$ 190 (4)	4600 (21); 4300 (25)	4.6; 4.3

female rat the urinary excretion of the amines faithfully reflects the rate of endogenous amine production. Thus, determinations of urinary excretion of amines seem to be a useful means to follow daily fluctuations in amine formation.

In rat pregnancy high levels of diamine oxidase activity have been found in the

Fig. 4. Urinary excretion (n-mole/24 hr) of diamines and polyamines in four rats (nos. 8 (■), 9 (▨), 10 (▧) and 11 (▩)) before, during and after pregnancy under the influence of aminoguanidine (10 mg aminoguanidine hemi-sulphate/kg once daily). Rat no. 8 and rat no. 10 were delivered of one and thirteen young by Caesarian section on the 25th and 24th day of pregnancy, respectively. Rat no. 9 gave birth to thirteen young on the 23rd day of pregnancy and rat no. 11 bore ten young on the 24th day of pregnancy. × indicates the day of parturition. ● indicates sample lost.

uterus and placenta as well as in the plasma (Roberts & Robson, 1953; Kobayashi, 1964). None the less, substantial amounts of diamines appeared unmetabolized in the urine in undisturbed pregnancy. To obtain a more precise measure of the actual amine formation as reflected in the urinary excretion, the break-down via the diamine oxidase pathway was inhibited by aminoguanidine.

Diamine oxidase was initially thought to function as a histaminase. Later it was shown that the preferred substrates for the enzyme are aliphatic diamines such as putrescine and cadaverine (for references see Zeller, 1965; Bardsley, Hill & Lobley, 1970). The results of the present study are compatible with these findings. During the administration of aminoguanidine the increase in the urinary excretion of putrescine and cadaverine was more pronounced than that of histamine. This is reflected both in the elevation of the urinary excretion of these diamines in the non-pregnant animals and in the elevation of the basal excretion and peak values of the pregnant animals.

Methylhistamine is quantitatively an important metabolite in histamine degradation. The methods available for determination of this metabolite have been complicated and time consuming and our knowledge of the excretion of methylhistamine during pregnancy is fragmentary. However, the step-wise elution technique of the liquid chromatographic method used in the present study makes it possible to determine methylhistamine as well as diamines and polyamines in one and the same analysis of a sample. In the non-pregnant female rat given aminoguanidine the excreted methylhistamine fraction has been shown to be of the same magnitude as that of histamine (Wetterqvist & White, 1968). This is confirmed in our study. Further, it is shown that even without administration of aminoguanidine appreciable amounts of methylated histamine appeared in the urine. During the latter part of pregnancy the elevation of urinary histamine excretion was accompanied by a corresponding increase in methylhistamine excretion. Thus, it appears that the fetus is capable of forming not merely large amounts of histamine but also to methylate a large portion of the newly formed amine as suggested by Lindberg, Lindell & Westling (1963).

In contrast to the great number of investigations of histamine and putrescine metabolism in various physiological connexions our knowledge of cadaverine in mammalian tissue is very scarce. It is known that rats regularly excrete cadaverine and the amine has also been found in human urine (Bremer & Kohne, 1971). Evidence for biosynthesis of cadaverine from lysine in mammalian tissue was originally reported following the administration of the anabolic steroid nandrolone (Henningsson *et al.* 1976). Even in tissues of the pregnant rat *in vitro* formation of cadaverine has been observed (to be published). The elevated excretion of cadaverine during the last week of pregnancy found in the present study is thus another example of elevated diamine formation in relation to growth.

The urinary content of spermine was unchanged during pregnancy, while the spermidine excretion was elevated during the last week of pregnancy. Jänne, Raina & Siimes (1964) showed that in several tissues of new-born rats the molar ratio of spermidine to spermine was high and subsequently decreased with age. In this connexion it should be observed that several workers have reported a correlation between an increase in the ratio of spermidine to spermine and increased growth (see Russell,

1972; Heby, Marton, Wilson & Martinez, 1975). Interestingly, as we found in the rat, also in human pregnancy spermidine excretion has been reported elevated during the first week after term (Fujita, Nagatsu, Maruta, Ito, Senba & Miki, 1976), the reason for which is unknown.

The distinct changes in the excretion of diamines and their derivatives during the last trimester in rat pregnancy suggest a possible relation between changes in diamine and polyamine metabolism and the hormonal status of pregnancy. Also in human pregnancy the diamine and polyamine excretion has been reported elevated (Russell, Levy, Schimpff & Hawk, 1971; Granerus, Gillbrand & Wetterqvist, 1977). Our results imply that diamine formation is important during the period of rapid growth of the fetus. The exact cause of the increased output of amines in the pregnant rat will be further studied in order to shed light on the involvement of diamine and polyamine metabolism in female reproductive physiology.

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ON THE BIOGENESIS OF DIAMINES AND POLYAMINES IN THE
PREGNANT RAT

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ABSTRACT

The urinary excretion of the diamines histamine, methylhistamine, putrescine and cadaverine and the polyamines spermidine and spermine was studied in rats which on the 19th day of pregnancy were subjected to various ectomizing operations.

In sham-operated rats the urinary excretion of all the amines studied except spermine was highly elevated on the day preceding the sham-operation and on the three days studied postoperatively, i.e. sham-operation did not affect the elevated urinary amine excretion during pregnancy.

Fetectomy resulted in an abolished increase in the urinary excretion of histamine and methylhistamine while the excretion of putrescine, cadaverine and spermidine was still significantly increased.

Removal of both the fetuses and placentae reduced the excretion of putrescine, cadaverine and spermidine towards the level of non-pregnant rats.

Combined hysterectomy and ovariectomy did not cause any additional effects to those after removal of the fetuses and the placentae except for cadaverine, the excretion of which was further reduced.

Previous work linking histaminase and the diamine histamine to a normal physiological process, i.e. pregnancy (Danforth & Gorham 1937; Marcou et al. 1938; Roberts & Robson 1953; Kahlson et al. 1958a), has recently been broadened to comprise some other biogenic amines, namely the diamines putrescine and cadaverine and the polyamines spermidine and spermine (Andersson et al. 1978). The results, showing that in the rat there occurred a striking elevation in the urinary diamine and polyamine excretion during the last trimester, strengthened the idea of a relation between changes in diamine metabolism and the state of pregnancy.

This idea is supported by experiments showing that administration of α -difluoromethyl ornithine, an irreversible inhibitor of ornithine decarboxylase (Metcalf et al. 1978), to mice during pregnancy resulted in a greatly retarded fetal development followed by resorption and/or expulsion of the fetuses (Fozard et al. 1979). The period when administration of α -difluoromethyl ornithine displayed contragestational effects corresponded exactly to a period of large rises in uterine ornithine decarboxylase activity and amine content. These findings provided strong evidence that changes in diamine metabolism are essential for the continuation of normal murine embryogenesis (Fozard et al. 1980).

Under the influence of the diamine oxidase inhibitor aminoguanidine the excreted amounts, particularly of putrescine and cadaverine, were greatly increased, indicating that oxidative deamination is a major route of catabolism of these compounds in rats (Andersson et al. 1978; Rojansky et al. 1979; Andersson et al. 1981).

A considerable number of observations have shown a correlation between enhanced synthesis of putrescine and polyamines and rapid growth or cell proliferation (for ref. see Tabor & Tabor 1976; Jänne et al. 1978). An increased diamine formation, i.e. formation of putrescine (Guha & Jänne 1976a) and cadaverine (Andersson et al. 1979), has been shown in the ovary of the pregnant rat. In addition, a high putrescine (Maudsley & Kobayashi 1977) and cadaverine (Andersson et al. 1979) formation has been found in the rat placenta.

The increased histamine formation during rat pregnancy, as reflected by the urinary excretion of the mother, was localized to the fetuses. It was shown that after removal of the fetuses before term the increased urinary excretion of histamine was rapidly abolished (Kahlson et al. 1958b). In addition the fetuses showed a high rate of histamine formation in vitro.

In the present study the influence of various ectomizing operations was studied to throw light on the origin of the elevated urinary excretion of diamines and polyamines in the pregnant rat.

METHODS

Animals

Adult female rats, weight 210-240 g, of the Sprague-Dawley strain, were used. They were fed a semi-synthetic diet, 18-27 g (dry weight) daily and water ad libitum. The composition of the diet has been given by Kahlson et al. (1958a) and its content of diamines and polyamines by Andersson et al. (1978). The oestrus cycles of the rats were followed by vaginal cytology; smears were taken in the morning and stained with methylene blue. Rats meant to become pregnant were mated in the pro-oestrus or early oestrus phase and were considered pregnant if spermatozoa were found in the vaginal smear and/or a vaginal plug were found the next morning. This day was termed day one of pregnancy.

The rats were divided into two groups: A = rats not given the diamine oxidase inhibitor aminoguanidine (AMG), i.e. non-AMG treated rats, B = rats treated with AMG (10 mg AMG hemi-sulphate/kg daily during pregnancy). Non-pregnant rats were treated with AMG for a corresponding period of time. From day one of pregnancy the rats were kept in metabolism cages for every second day for training. On the 7th day of pregnancy when the urinary excretion of diamines and polyamines is still at non-pregnant level urine was collected. Another urine sample was collected on the 18th day of pregnancy, during the period of highly elevated levels of urinary diamines and polyamines (Andersson et al. 1978). On the 19th day of pregnancy various

operative procedures were performed and after completed surgery urine was collected for three consecutive days.

Surgery

In non-AMG treated as well as AMG treated rats five types of operations (I-V) were performed. Groups I-IV consisted of pregnant animals while the rats of the last group were non-pregnant. Each group consisted of four animals. All surgery was carried out under ether anesthesia.

I. The pregnant rats of the first group were subjected to sham-operations on the 19th day of pregnancy. A midline incision was made, through the skin and abdominal muscles. The gravid uterine horn of one side was gently exposed and carefully examined. Thereafter it was put back into its proper place and the uterine horn of the other side was examined. Then the abdominal wall was closed with silk sutures, first the muscular layer and then the skin. The duration of these procedures were adjusted to correspond to those of groups II-V. The sham-operated rats gave birth to their young (non-AMG treated rats: 7-14 young, AMG treated rats: 12-17 young) on the 23rd day of pregnancy.

II. In these pregnant rats the fetuses were removed (non-AMG treated rats: 11-17 fetuses, AMG treated rats: 15-17 fetuses) while the placentae were left intact. The abdominal cavity was opened and a uterine horn was exposed. A small incision, parallel with the longitudinal axis of the uterine muscle fibers, was made on the antimesometrial side of the uterine wall. As the amniotic sac was opened the amniotic fluid was absorbed. The fetus was gently pushed out and the umbilical cord was cut. Then the incision was closed with silk sutures. These procedures were repeated for each fetus. Great care was taken not to dislodge the placentae. Before closure of the abdominal wall remaining amniotic fluid was absorbed from the abdominal cavity. These rats which can be considered as physiologically pregnant (Kirsch 1938; Selye et al. 1935), delivered their placentae on the normal full-term date.

III. In the third group of pregnant rats the fetuses as well the placentae (non-AMG treated rats: 13-14 fetuses,

AMG treated rats: 10-14 fetuses) were removed through the incision in the uterine muscle. The amniotic sac was kept intact during the removal and amniotic fluid was collected for analysis of its content of diamines and polyamines.

IV. These pregnant rats were studied after removal of both the gravid uterus and the ovaries. First the placentae and the fetuses (non-AMG treated rats: 10-15 fetuses, AMG treated rats: 13-16 fetuses) were removed to strangle the heavy uterine circulation. Then the ovarian and uterine vessels were ligated and the uterus and the ovaries were excised. Amniotic fluid was collected also from rats subjected to this kind of surgery.

V. The non-pregnant rats of the fifth group were subjected to a combined hysterectomy and ovariectomy, which was performed similarly as described for the pregnant rats of group IV.

Care was taken to maintain clean, though not sterile operation conditions. In addition, care was taken to keep the animals warm and comfortable during the postoperative recovery period. During the three days of urine collection after surgery a fan was put on to keep them warm in the metabolism cages. To promote postoperative recovery 4 ml 5.5 % (w/v) glucose was given sc at the awakening of the narcosis and later the same day 4 ml 0.9 % (w/v) NaCl (saline) was injected sc. On the first day postoperatively these injections were repeated and on the next day 4 ml saline was given. The rats recovered quickly and 60-90 min after completed surgery they were reinstalled in their metabolism cages.

Assay of diamines and polyamines in the urine

Urine was collected with the rats in metabolism cages which provided facilities for collection of urine separately from faeces. The urine was collected in vessels containing hydrochloric acid sufficient to give the urine a pH value below 2 to prevent bacterial formation and/or destruction of the amines during the 24 hr collection periods. After rinsing the collecting equipment with distilled water, the volume of the sample was noted whereafter it was filtered

through a Munktell's filter paper No. V20H. The urine was then stored at -18°C until an aliquot was prepared for analysis of its diamine and polyamine content. For this preparation 1 mg EDTA (ethylene diamine tetraacetic acid) and 30 mg sulfosalicylic acid were added to each ml of urine. The samples were then centrifuged at $800 \times g$ for 15 min whereafter the supernatant was adjusted to a pH value of 2.0-2.5 and filtered through a Millipore filter ($0.22 \mu\text{m}$). The separation and quantitative estimation of the diamine and polyamine content in the urine were carried out on a 6×78 mm column of Durrum DC 6A by the means of the automated amino acid analyser LKB-BIOCAL 3201. The method described by Henningsson (1978) was used with minor modifications (Andersson et al. 1978).

Assay of diamines and polyamines in amniotic fluid

Amniotic fluid was collected from non-AMG as well as AMG treated rats of groups III and IV. To each ml of amniotic fluid 0.1 % (w/v) sulfosalicylic acid was added. The samples were then stored at -18°C until prepared for analysis of its content of diamines and polyamines. For the preparation 1 mg EDTA was added per ml of sample. The subsequent preparation and assay were carried out as described above for urine samples.

RESULTS

The urinary excretion of histamine, methylhistamine, putrescine, cadaverine, spermidine and spermine was determined in rats subjected to various kinds of surgery on the 19th day of pregnancy. The urinary content of the six amines was determined in samples from the 7th day of pregnancy, the day before surgery and for three days post-operatively.

The influence of various ectomizing operations on the urinary excretion of diamines and polyamines

Histamine and methylhistamine. As seen in Fig. 1 the urinary excretion of histamine and methylhistamine in the sham-operated animals (I) was highly elevated on the day

preceding the sham-operation and on the three days studied postoperatively, i.e. sham-operation did not interfere with the earlier reported elevated excretion of these amines during the last trimester of rat pregnancy. In Fig. 1 it is also confirmed that administration of aminoguanidine further elevated the urinary excretion of these amines. As to the site of formation of histamine Kahlson et al. (1958b) showed that fetectomy abolished the increased urinary excretion of histamine in the pregnant rat. In the

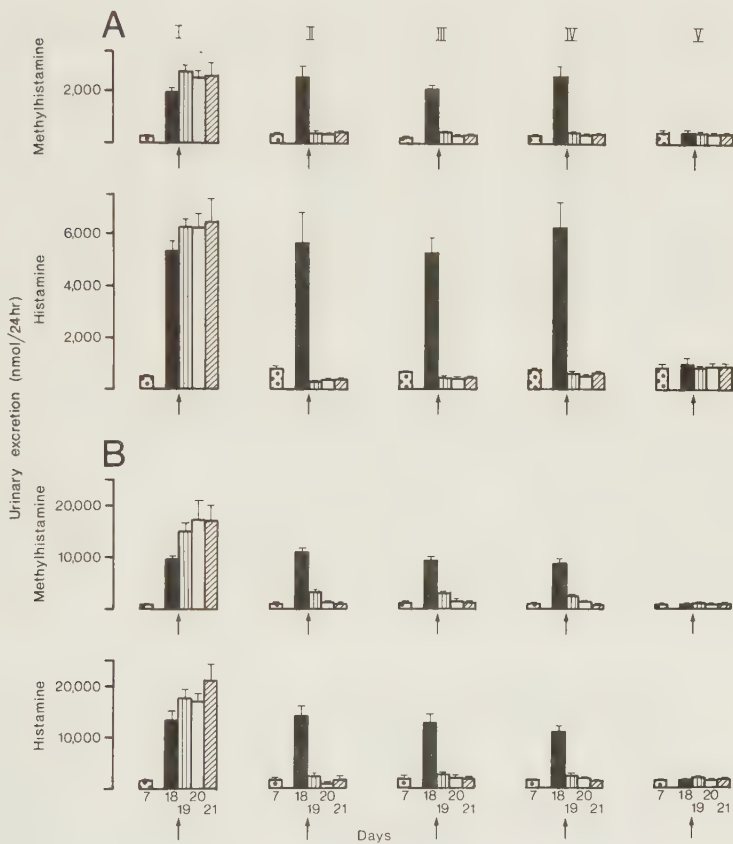


Fig. 1. Urinary excretion (nmol/24 hr) of methylhistamine and histamine in non-AMG (A) and AMG treated (B) rats subjected to various ectomizing operations. Group I-IV: pregnant rats, group V: non-pregnant rats. The arrow denotes the time of surgery. I=sham-operation; II=fetectomy; III=fetectomy + placentectomy; IV=removal of the gravid uterus + ovaries; V=hysterectomy + ovariectomy. Each column represents the mean value $\pm$ S.E. of mean of determinations from four rats.

present study this result was confirmed and was shown also to apply to methylhistamine. As seen in Fig. 1 removal of the fetuses (II) reduced the excretion of histamine as well as methylhistamine towards the level of non-pregnant rats. No further change in the urinary excretion of methylhistamine and histamine was attained by removing also the placentae and/or the uterus and the ovaries (III, IV). Essentially the same pattern of excretion occurred in the rats whether they had received AMG or not, though the non-pregnant level of urinary excretion of methylhistamine was reached in a more step-wise manner in the AMG treated rats.

There was no change in the urinary excretion of methylhistamine and histamine in the non-pregnant rats subjected to the combined hysterectomy and ovariectomy (V).

Putrescine and cadaverine. From Fig. 2 it is obvious that the content of putrescine and cadaverine in the urine was greatly increased on the day before the sham-operation and on the three days postoperatively (I), i.e. sham-operation did not change the pattern of the elevated urinary excretion of putrescine and cadaverine earlier reported during late pregnancy in rats. It is also confirmed that administration of aminoguanidine highly elevated the excreted amounts of putrescine and cadaverine. Removal of the fetuses on the 19th day of pregnancy did not reduce the excretion of these diamines to non-pregnant levels (II). The urinary excretion of putrescine was reduced, though still significantly ($p < 0.001$) above the non-pregnant level, during the first day postoperatively in the non-AMG treated rats. It increased during the following two days and there was no significant difference between the urinary excretion of putrescine of the sham-operated and fetectomized animals during the second and third day after the operations. In the AMG treated animals there was a step-wise decrease in the putrescine excretion during the three days following fetectomy but the excretion was still four times the non-pregnant level on the third day postoperatively. Removal of the fetuses as well as the placentae (III) significantly ($p < 0.001$ versus sham-operated animals)

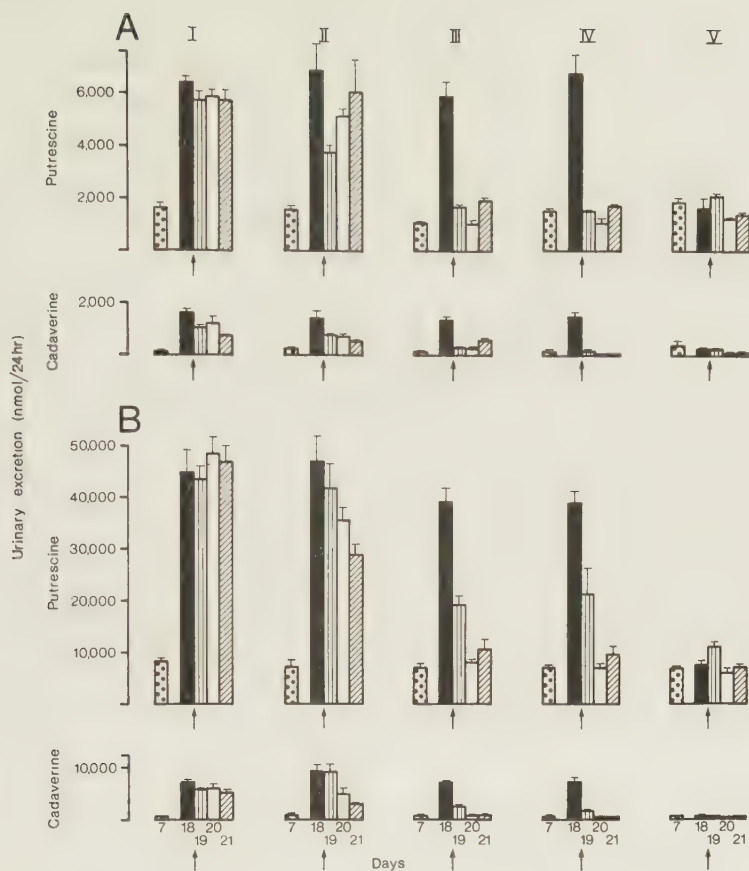


Fig. 2. Urinary excretion (nmol/24 hr) of putrescine and cadaverine in pregnant (I-IV) and non-pregnant (V) rats before and after various ectomizing operations. For details see Fig. 1.

reduced the urinary excretion of putrescine. This can be seen both in the non-AMG treated rats and in the rats treated with AMG though the decrease in the putrescine excretion was smaller on the first day postoperatively in the AMG treated rats. Hysterectomy and ovariectomy of the pregnant rats (IV) did not further change the urinary excretion of putrescine.

The urinary excretion of cadaverine after fetectomy was still significantly higher than the level of non-pregnant rats while it was significantly ($p < 0.001$ versus sham-operated animals) reduced by the removal of also the

placentae. The combined hysterectomy and ovariectomy of the pregnant rats further depressed the urinary excretion of cadaverine. The same pattern of urinary excretion of cadaverine was seen in non-AMG as well as AMG treated animals.

There were small but not significant fluctuations in the urinary excretion of putrescine and cadaverine following hysterectomy and ovariectomy of non-pregnant rats (V).

Spermidine and spermine. As to the excretion of spermidine (Fig. 3) the same changes were seen in non-AMG and AMG treated rats, but the more pronounced elevations of spermidine excretion of the pregnant rats treated with AMG made these rats more suitable for evaluating the effects of the ectomizing operations. Sham-operation (I) or fetectomy (II) did not depress the excretion of spermidine to the non-pregnant level. In the rats subjected to type III and IV operations the urinary spermidine excretion of the first day following surgery was still at a high level but there was a significant ($p < 0.001$ versus sham-operated

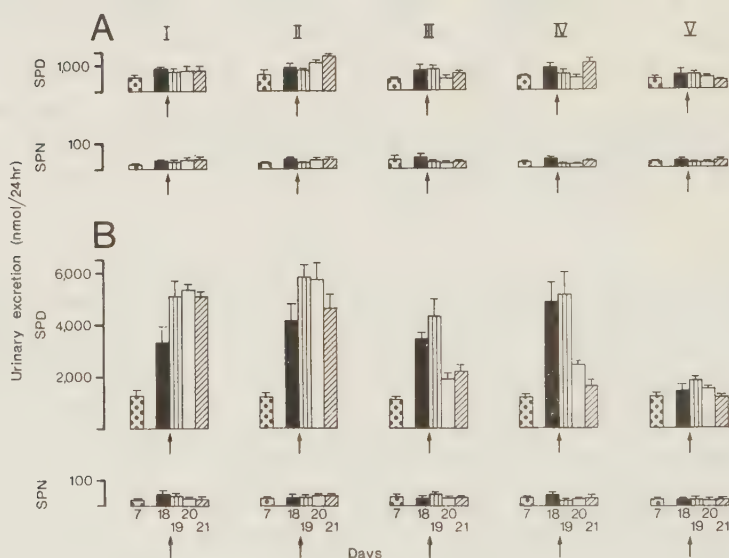


Fig. 3. Urinary excretion (nmol/24 hr) of spermidine (SPD) and spermine (SPN) in pregnant (I-IV) and non-pregnant (V) rats subjected to various ectomizing operations. For details see Fig. 1.

animals) reduction of the excretion of spermidine on the second and third day postoperatively.

The combined hysterectomy and ovariectomy of the non-pregnant rats did not cause any significant changes in the urinary spermidine content.

The urinary excretion of spermine was much lower than that of any of the other amines studied. Neither pregnancy, treatment with AMG nor surgery caused any significant change in the urinary excretion of spermine (Fig. 3).

Diamines and polyamines in amniotic fluid

As seen in Table 1 histamine, putrescine, cadaverine, spermidine and spermine were found in the amniotic fluid of pregnant rats, while methylhistamine was not detectable. Putrescine was the amine found in the highest concentration (20 nmol/ml). There was no significant difference in the diamine and polyamine content of the amniotic fluid of non-AMG and AMG treated animals, indicating the possibility that AMG did not pass into the amniotic fluid.

DISCUSSION

During the last third of pregnancy the rat produces large amounts of diamines and polyamines, as indicated by their excretion in the urine (Kahlson et al. 1958a; Andersson et al. 1978; Rojansky et al. 1979).

Table 1. Content of diamines and polyamines (nmol/ml) in amniotic fluid. A: non-AMG treated rats, B: rats treated with AMG. The figures are mean values $\pm$ S.E. of mean of four determinations. 0 stands for not measurable amount.

	A	B
Methylhiatamine	0	0
Histamine	8.0 $\pm$ 1.68	6.4 $\pm$ 0.81
Putrescine	22 $\pm$ 0.68	18 $\pm$ 3.06
Cadaverine	2.8 $\pm$ 0.25	2.7 $\pm$ 0.49
Spermidine	8.1 $\pm$ 1.45	6.7 $\pm$ 1.44
Spermine	1.2 $\pm$ 0.14	1.0 $\pm$ 0.18

Kahlson et al. (1958b) showed that the increased histamine excretion of the mother was of fetal origin. Removal of the fetuses before term rapidly abolished the increased histamine excretion of the mother, a finding which was confirmed in the present study. In addition, corresponding results were obtained for methylhistamine, an important metabolite in the degradation of histamine.

Removal of the fetuses with the placentae left intact did not abolish the increased urinary excretion of putrescine, cadaverine and spermidine, indicating that the presence of the placentae, but not the fetuses, was of crucial importance for the elevated output of these amines. In non-AMG treated rats, after fetectomy, there was a decrease in the urinary content of putrescine on the first day after the operation followed by a rise during the next two days. These changes may be caused by alterations in the activities of ornithine decarboxylase and/or diamine oxidase as a consequence of the removal of the fetuses. No corresponding rise occurred in the excretion of cadaverine, a finding which might be explained by a more efficient catabolism of cadaverine as this diamine has been shown to be a better substrate for diamine oxidase (Bardsley et al. 1970).

The gradual decrease in the urinary excretion of both putrescine and cadaverine seen in the fetectomized rats after administration of AMG might be explained by a gradual deterioration of the synthesis of these amines after fetectomy.

Removal of the placentae, changes the hormonal status prevailing during pregnancy. Therefore the abolished increase in the urinary excretion of putrescine, cadaverine and spermidine following removal of the fetuses and the placentae does not necessarily indicate that these amines are of placental origin. However, the site of cadaverine formation in the pregnant rat has been investigated by a more direct approach (Andersson et al. 1979). Of the various tissues examined the fetal placenta displayed the highest in vitro formation of cadaverine. This finding suggests that the elevated urinary excretion of cadaverine

during pregnancy is caused by the high rate of cadaverine formation in the fetal placenta. The same may be valid for putrescine as the placenta has been shown to possess a high capacity of forming putrescine (Guha & Jänne 1976b; Maudsley & Kobayashi 1977).

In the rat not allowed to suckle her young the urinary excretion of spermidine displayed a biphasic course with one peak before parturition and another occurring after delivery (Andersson et al. 1978). Preliminary results show that in lactating rats the excretion of spermidine was prolonged and augmented (Andersson & Henningsson, unpublished). In the blood the spermidine level has been found to be increased during lactation (Lundgren & Oka 1978). The enzymes responsible for the synthesis of spermidine (ornithine decarboxylase and S-adenosyl-L-methionine decarboxylase) have been reported to be highly elevated in the mammary gland during pregnancy and lactation and in addition the content of spermidine was found strikingly high during these conditions (Neish & Key 1968; Russell & McVicker 1972). These findings make it possible that the mammary gland might be one source of the elevated amount of spermidine found in the urine of the pregnant rat. The relevance of this suggestion might be clarified by studying the urinary spermidine excretion after the excision of the mammary glands during pregnancy. In addition substitution with relevant hormones of rats after removal of fetuses and placentae may afford further information on the relation between diamine and polyamine metabolism and female reproduction.

ACKNOWLEDGMENTS

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Formation of cadaverine in the pregnant rat

By

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Abstract

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The formation as well as the content of cadaverine were determined in different tissues of pregnant and non-pregnant rats. The placenta and ovary were most potent in the ability to form cadaverine. To our knowledge this is the first report of an in vitro formation of cadaverine linked to a normal physiological process, i.e. pregnancy. The highest concentration of cadaverine was found in the placenta and ovary of the pregnant rat. Treatment with aminoguanidine generally elevated the content of cadaverine, indicating a role of diamine oxidase as a regulator of diamine content.

Key words: Cadaverine formation, content of cadaverine, rat pregnancy

Evidence of in vitro formation of cadaverine in mammalian tissue was originally reported in the mouse kidney after the injection of the anabolic steroid Durabolin (Henningsson, Persson and Rosengren 1976). An in vivo formation of cadaverine is supported by the observation that rats regularly excrete cadaverine in the urine. The amine has also been detected in human urine (Bremer and Kohne 1971). In the pregnant rat the urinary excretion of cadaverine together with other diamines as well as the polyamine spermidine is greatly increased (Andersson, Henningsson and Rosengren 1978). On the 15th day of pregnancy the cadaverine excretion began to rise and hence increased continuously to day 19-20 of pregnancy when peak values up to 10 times the non-pregnant level were observed. Thereafter the cadaverine excretion fell towards the non-pregnant level which after the birth of the young was resumed.

The purpose of the present study was to investigate in which tissue(s) of the pregnant rat an increased formation of cadaverine takes place. In addition the content of cadaverine was determined in various tissues of the pregnant and non-pregnant rat. As stated above, very little is known about the synthesis of cadaverine in mammalian tissues. Regarding the close congener of cadaverine, i.e. putrescine, it has been shown that its synthesis is controlled by the level of the amine itself in different cell culture systems and in intact animals (for ref. see Jänne, Pösö and Raina 1978). The diamine oxidase inhibitor aminoguanidine might a

priori increase the level of tissue cadaverine and such an effect might influence its synthesis. The diamine oxidase activity is known to be high in the placenta and plasma of pregnant rats (Roberts and Robson 1953, Kobayashi 1964).

Methods

Adult female rats, weight 240–340 g, of the Sprague-Dawley strain were used. They were fed a standard pellet diet and water ad libitum. Their estrus cycles were followed by vaginal cytology. Smears were taken in the morning and stained with methylene blue. Only rats with normal estrus cycles were used. Rats selected to become pregnant were allowed to mate in the pro-estrus or early estrus phase. They were then transferred to a male for 24 h. The next morning when sperm was found in the vaginal smear was considered as the first day of pregnancy. On the 19th day of pregnancy the rats were killed by cervical dislocation and exsanguinated. Concomitantly control rats were sacrificed. One group of rats was studied under undisturbed conditions and another group after the injection of the diamine oxidase inhibitor aminoguanidine (10 mg aminoguanidine hemisulphate/kg) for 10 consecutive days before the actual experiment.

Tissues of pregnant rats (ovary, uterus, kidney, liver, fetus and placenta) and of non-pregnant rats (ovary, uterus, kidney and liver) were rapidly removed, cooled on ice, minced with scissors and gently homogenized in a Dounce type homogenizer in 4 volumes of cold 0.1 M sodium phosphate buffer (pH 7.2) containing 5×10^{-4} M dithiothreitol, 10^{-4} M EDTA and 2% (w/v) glucose. The homogenate was centrifuged at $20\,000 \times g$ for 20 min at 4°C. The supernatant was used as enzyme source. Supernatant corresponding to 160 mg of tissue (uterus, kidney, liver and fetus), 60 mg of tissue (placenta in its whole, maternal and fetal forms) and 16–57 mg (ovary) was incubated in the presence of pyridoxal-5-phosphate (final conc. 10^{-3} M), DL-(1- 14 C)-lysine monohydrochloride (final conc. 10^{-2} M, sp. act. 10 μ Ci/mmol) and the same buffer as used for homogenization, the total finally made up to 1.0 ml. The incubation was carried out at 37°C for 60 min with agitation. The reaction was stopped by the tipping of 0.7 ml of 2 M citric acid from a side arm of the incubation vessel. Expelled $^{14}\text{CO}_2$ was trapped on a 10×25 mm piece of No. 005 Munktell's filter paper prepared with 100 μ l hydroxide of Hyamine (1 M solution in methanol). For maximal absorption of the $^{14}\text{CO}_2$ the shaking was continued for another 45 min. After completed incubation the filter paper was dropped into a scintillation vessel containing 8 ml of scintillation solution (Bray 1960) and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

For stoichiometric determinations of cadaverine formation supernatants of the placenta and ovary were incubated in the presence of aminoguanidine in a final concentration of 2×10^{-5} M to prevent oxidative deamination of the formed cadaverine by diamine oxidase. The reaction was stopped by the tipping of 0.7 ml 9.7% sulfosalicylic acid from the side arm of the incubation vessel. The amount of $^{14}\text{CO}_2$ expelled was compared with the amount of the cadaverine formed in the same incubate. For the preparation of the sample for analysis of its cadaverine content 0.4 mg EDTA was added to each ml of the incubate. The incubates were then centrifuged at $800 \times g$ for 15 min whereafter the samples were adjusted to a pH value of 2.0–2.5 and filtered through a filter with a pore size of 0.22 μ m (Millipore Corp.). The separation and quantitative estimation of cadaverine in the incubates were carried out on a 6×78 mm column of Durrum DC 6A using an automatic amino acid analyzer (LKB-BIOCAL, 3201). The procedure as described by Henningsson (1978) was used with minor modifications.

For determination of the content of cadaverine in the tissues of pregnant and non-pregnant rats about 200 mg of tissue was homogenized in a Duran 50 type homogenizer in 3.5 ml sulfosalicylic acid (40 mg sulfosalicylic acid and 0.4 mg EDTA/ml). The extracts were then boiled on a water-bath for 30 min, whereafter they were prepared and assayed in the same way as described above for determination of cadaverine content in incubates.

Results

Cadaverine formation in various tissues from pregnant and non-pregnant rats

The formation of cadaverine in vitro in various tissues from pregnant rats was examined at times when, from an earlier investigation (Andersson *et al.* 1978), the urinary excretion of

TABLE I. Formation of cadaverine (nmol/g $\times$ h) in different tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of pregnancy. The figures are mean values $\pm$ S.E. of mean of 8 observations (tissues from 4 untreated rats and 4 rats treated with aminoguanidine).

Tissue	Non-pregnant	Pregnant
Ovary	25 $\pm$ 7.47	110 $\pm$ 16.3***
Uterus	22 $\pm$ 3.40	9.0 $\pm$ 1.49**
Kidney	2.1 $\pm$ 1.03	3.9 $\pm$ 1.38
Liver	2.7 $\pm$ 2.11	1.8 $\pm$ 1.01
Fetus		5.9 $\pm$ 1.94
Whole placenta		250 $\pm$ 20.9
Fetal placenta		350 $\pm$ 29.0
Maternal placenta		52 $\pm$ 10.0

** = $p < 0.01$ and *** = $p < 0.001$ versus non-pregnant.

cadaverine is known to be high, *i.e.* on the 19th day of pregnancy. In addition, determinations were made on tissues of non-pregnant female rats. The tissues were collected from two groups of rats of which one group was treated with aminoguanidine as states above, the other was not. Since no difference was found between the values of the two groups, the figures are brought together and presented in Table I from which it will be seen that of the tissues examined the placenta was most potent in the ability to form cadaverine, followed by the ovary and the uterus. As to the uterus the activity was higher in that of the non-pregnant rat than in that of the pregnant one. This may be attributed to the fact that the activity of cadaverine formation is expressed in nmol per g wet weight of the tissue. It is known that the pregnant uterus is edematous and for that reason the total activity of the organ may not be reduced during pregnancy. Both kidney and liver showed low cadaverine formation and no differences between pregnant and non-pregnant rats were observed.

The question arose as to which part of the placenta, the maternal one and/or the fetal one, contributed to the synthesis of cadaverine. For that reason the organ was macroscopically dissected and the enzyme activity of the maternal and fetal parts was determined. It was found that the enzyme responsible for the formation of cadaverine was mainly located in the fetal placenta, in fact the synthesis of cadaverine in the maternal part was only 15% of that in the fetal one (Table I).

TABLE II. Stoichiometric determinations of cadaverine formation in tissues of the pregnant rat. Parallel assays of $^{14}\text{CO}_2$ production and formation of cadaverine (nmol/g $\times$ h) were carried out in the same incubate.

	$^{14}\text{CO}_2$	Cadaverine
Whole placenta	340	340
	280	330
	220	180
	200	170
Fetal placenta	410	450
	340	440
Ovary	64	50
	43	39

TABLE III. Content of cadaverine (nmol/g) in different tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of pregnancy. The figures are mean values $\pm$ S.E. of mean of 4 determinations, except for those indicated with (a) which stands for 3 determinations. A: untreated rats, B: rats treated with aminoguanidine. 0 stands for not measurable amount.

Tissue	Pregnant		Non-pregnant
	A	B	B
Ovary	14 $\pm$ 4.75	28 $\pm$ 5.04	4.1 $\pm$ 2.62**
Uterus	3.9 $\pm$ 2.44	5.3 $\pm$ 1.78	2.7 $\pm$ 1.98
Kidney	0	23 $\pm$ 3.10	32 $\pm$ 2.71*
Liver	7.6 $\pm$ 3.00	14 $\pm$ 4.22	7.9 $\pm$ 0.43
Fetus	3.3 $\pm$ 3.25	11 $\pm$ 1.61 (a)	
Whole placenta	10 $\pm$ 1.43	14 $\pm$ 2.42	
Fetal placenta	13 $\pm$ 0.88	15 $\pm$ 1.20 (a)	
Maternal placenta	9.5 $\pm$ 2.05	20 $\pm$ 3.28 (a)	

* = $p < 0.05$ and ** = $p < 0.01$ versus pregnant rats treated with aminoguanidine.

Parallel assays of $^{14}\text{CO}_2$ -production and the formation of cadaverine from DL-(1- ^{14}C)-lysine were performed to ensure that the evolution of $^{14}\text{CO}_2$ provided a valid measure of the biosynthesis of cadaverine. Values of cadaverine formation in the placenta and the ovary are shown in Table II from which it is apparent that there was a fair accordance in results obtained by the two methods showing that artifactual release of $^{14}\text{CO}_2$ did not occur.

Content of cadaverine in various tissues from pregnant and non-pregnant rats

The cadaverine concentration in tissues of pregnant rats was determined. Of these rats one group had been injected with aminoguanidine for 10 days before the actual experiment, the other group was untreated. The results are summarized in Table III in which also results from non-pregnant rats given aminoguanidine are presented. In the untreated pregnant rat most cadaverine was found in the ovary and placenta. Aminoguanidine treatment of the animals generally elevated the cadaverine concentration of the tissues. The most distinct increase was seen in the kidney, an organ known to be a source of diamine oxidase. Also in the non-pregnant group the highest concentration of cadaverine was found in the kidney. Notable is the significant difference in the cadaverine content of ovary between the pregnant and non-pregnant animals, a result which might reflect the high synthesis in the ovary of the pregnant rat.

Discussion

Although it has been known for some time that cadaverine is regularly excreted in the urine its origin is uncertain. Most authors regard the amine as a contaminant believed to be formed by bacteria in the lumen of the intestine, absorbed and carried by the blood to tissues and urine. However, some recent findings strongly suggest that cadaverine can be synthesised in mammalian tissues both in vivo and in vitro. Cadaverine has been found in the brain and blood of germ-free mice (Stepita-Klauco and Dolezalova 1974). An in vitro formation of cadaverine in mouse kidney has been observed after the injection of the anabolic steroid

Durabolin (Henningsson *et al.* 1976). In the present investigation an in vitro formation of cadaverine was linked to a normal physiological process, *i.e.* pregnancy.

The high formation of cadaverine in the fetal part of the placenta is reflected in an elevated excretion of cadaverine during the last trimester of rat pregnancy (Andersson *et al.* 1978). This circumstance is supported by experiments carried out on the 19th day of pregnancy, showing that after the removal of the fetuses without dislodging the placentas a high urinary excretion of cadaverine was maintained, while on removing also the placentas the increased cadaverine excretion was abolished (to be published).

The elevated in vitro formation of cadaverine in the ovary during pregnancy is consistent with an increased endogenous cadaverine content of the organ indicating that cadaverine can be formed in vivo by decarboxylation of lysine.

Our present results together with our earlier findings of a regular pattern of urinary cadaverine excretion during pregnancy and the observation that cadaverine was formed in the mouse kidney stimulated to growth by an anabolic steroid strongly support our opinion that cadaverine is formed by mammalian tissue cells, and is not the product of bacterial decarboxylation of lysine.

Aminoguanidine treatment of the animals doubled the concentration of cadaverine in the maternal part of the placenta which is very rich in diamine oxidase. In the fetal part with a low content of diamine oxidase the change in cadaverine content was only slight. It appears conceivable that diamine oxidase could function as a regulator of cadaverine content in certain tissues rich in the enzyme. Another role of diamine oxidase in the maternal part of the rat placenta may be to protect the maternal tissues from diamines derived from the fetuses and/or the fetal part of the placentas.

The results presented here support our view of a connection between diamine metabolism and the hormonal status prevailing in pregnancy and are also another example of elevated diamine formation in relation to growth.

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Biosynthesis and accumulation of cadaverine and putrescine in rat ovary after administration of human chorionic gonadotrophin

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Abstract. In the ovaries of pre-pubertal rats stimulated by human chorionic gonadotrophin (hCG) the temporal changes in cadaverine and putrescine formation were investigated. In addition, the dose-response relationship of hCG and its effect on the diamine formation and the effect of hCG on the content of diamines and polyamines in the ovaries and the urine were studied. The results show that the ovary stimulated by hCG, in addition to putrescine, forms cadaverine at a highly increased rate. The elevated diamine formation was paralleled by an increased content of cadaverine and putrescine in the ovary.

Treatment with aminoguanidine elevated the content of cadaverine in the ovary, suggesting that diamine oxidase has a role as a regulator of the intra-ovary level of cadaverine.

These results confirm that cadaverine can be synthesized in an inducible manner in mammalian tissues. This is, virtually, the first report of elevated formation of cadaverine in response to an exogenous gonadotrophin.

In contrast to a great number of investigations on the metabolism of the diamine putrescine and the polyamines spermidine and spermine in various physiological connections (for ref. see Tabor & Tabor 1976; Jänne et al. 1978) our knowledge of the biosynthesis and metabolism of the diamine cadaverine in mammalian tissue is scarce. Evidence of an *in vitro* formation of cadaverine in eukaryotic cells was originally reported in the mouse kidney after the injection of the anabolic steroid nandrolone. The elevated formation of cadaverine was reflected by an increase in the urinary excretion of the amine (Henningsson et al. 1976). In the pregnant rat the urinary excretion of cadaverine as well

as putrescine and spermidine was greatly increased during the last week of gestation (Andersson et al. 1978a). *In vitro* studies showed that of the tissues investigated the placenta and the ovary were the most potent in the ability to form cadaverine (Andersson et al. 1979). These were the first reports where formation of cadaverine could be linked to a normal physiological process i.e. pregnancy.

These observations prompted us to study whether the exposure of rats to exogenous gonadotrophin would produce an increase in the formation of cadaverine in the ovary. Beside the pituitary gonadotrophins, polypeptides with gonadotrophic activity are present in body fluids during pregnancy in a number of species. The placenta is one of the sources of these gonadal-stimulating hormones. Chorionic gonadotrophin is a hormone characteristically found in primates during pregnancy. It is similar to luteinizing hormone (LH) in its main actions (Hamburger 1957). LH is considered to be the major ovulation inducing hormone in the rat. In the sequence of changes induced by this hormone several biochemical and morphological events have been described e.g. stimulation of steroidogenesis, resumption of meiosis in the oocyte and changes in the follicular wall leading to stigma formation and rupture of the follicle (Barraclough et al. 1971; Tsafiriri et al. 1972; Rondell 1974).

Elevated putrescine formation in the ovary of the rat after the administration of LH or human chorionic gonadotrophin (hCG) was first reported by Kobayashi et al. (1971).

In the present study the temporal changes in cadaverine and putrescine formation in response to hCG were examined in the ovaries of prepubertal rats. In addition, the dose-response relationship of hCG and its effect on the diamine formation and the effect of hCG on the content of diamines and polyamines in the ovaries and the urine were studied.

Materials and Methods

Animals. Twenty-eight-day-old rats of the Sprague-Dawley strain, bred at the institution, were used. After weaning at 23 days of age they were fed a diet of commercial rat chow and water ad libitum. Rats from which urine was collected were fed a partly synthetic diet for two days before and during the day of urine collection. The composition of the diet has been given by Kahlson et al. (1958) and its content of diamines and polyamines by Andersson et al. (1978a).

Hormone. hCG, human chorionic gonadotrophin (Gonadex®, Leo, Helsingborg, Sweden), was freshly dissolved in 0.9% NaCl (saline) and injected sc in a volume of 0.1 ml. Control rats received 0.1 ml saline sc.

Assay of in vitro formation of cadaverine and putrescine. Formation of cadaverine and putrescine was determined by estimating the amount of [^{14}C]O $_2$ evolved by decarboxylation of [^{14}C]carboxyl-labelled DL-lysine and [^{14}C]carboxyl-labelled DL-ornithine, respectively.

In the time-course experiments 50 IU of hCG was injected and the in vitro formation of cadaverine and putrescine was estimated in the ovaries at various intervals (Fig. 1) after the injection.

In the dose-response experiments the in vitro formation of cadaverine and putrescine was assayed in the ovaries 5 h after the injection of 1.5–200 IU of hCG.

The rats were killed by cervical dislocation and exsanguinated. In each determination, ovaries from 1–2 rats were rapidly removed, cooled on ice and weighed. Cold 0.1 M sodium phosphate buffer (pH 7.2), containing 5×10^{-4} M dithiothreitol, 10^{-4} M EDTA (ethylene diamine tetraacetic acid) and 0.2% (w/v) glucose, was added to a volume of 1.6 ml. The tissue was gently homogenized in a Dounce type of homogenizer (25 strokes with the pestle). The homogenate was centrifuged at $20\,000 \times g$ for 20 min at 4°C. The decanted supernatant was used for assay of cadaverine as well as putrescine formation.

For determination of cadaverine formation 0.8 ml of the supernatant, corresponding to 11–26 mg of tissue, was incubated in a reaction mixture (final volume 1.0 ml) containing 10^{-2} M [^{14}C]carboxyl-labelled DL-lysine monohydrochloride (sp. act. 100 $\mu\text{Ci}/\text{mmol}$) and 10^{-3} M pyridoxal-5-phosphate. To obtain low blank values the [^{14}C]

lysine solution was flushed with a gas mixture of 4% CO $_2$ and 96% N $_2$ for 30 min before use. The incubation was carried out at 37°C for 60 min with agitation. Determination of putrescine formation was carried out in an incubation mixture containing 0.1 ml of the supernatant (corresponding to 1.6–3.2 mg of tissue), 10^{-4} M [^{14}C]carboxyl-labelled DL-ornithine monohydrochloride (sp. act. 500 $\mu\text{Ci}/\text{mmol}$) and 10^{-5} M pyridoxal-5-phosphate, the total finally made up to a volume of 1.0 ml by the same buffer as used for homogenization. The incubation was carried out at 37°C for 30 min.

The reactions were terminated by the tipping of 0.7 ml 2 M citric acid from a side arm of the incubation vessel. Blanks were provided by incubating acidified samples. The expelled [^{14}C]O $_2$ was trapped on a 10×25 mm piece of Munktell's filter paper No. 005 prepared with 100 μl of hydroxide of Hyamine 10-X. Maximum absorption of the carbon dioxide formed during the incubation was achieved by continued shaking for another 45 min. The filter paper was then transferred to a counting vial containing 8 ml liquid scintillation solution (Bray 1960) and the radioactivity was assayed in a liquid scintillation spectrometer (Packard TRI-CARB 2002). The formation of cadaverine and putrescine was expressed in nmols per g wet weight and h. Protein concentration (estimated by the method of Lowry et al. (1951)) was unaffected by hCG administration.

Assay of diamines and polyamines in the ovaries. The content of the diamines cadaverine and putrescine and the polyamines spermidine and spermine was determined in the ovaries of rats at different times (Table 2) after the injection of 50 IU of hCG. Ovaries from 2–3 rats were pooled for each sample. The tissue was homogenized in a Duran 50 type of homogenizer in 2 ml sulfosalicylic acid (40 mg sulfosalicylic acid and 0.4 mg EDTA/ml). After boiling in a water-bath for 30 min the samples were centrifuged at $800 \times g$ for 15 min, adjusted to a pH value of 2.0–2.5 and filtered through a Millipore filter with a pore size of 0.22 μm . Until analysis of the diamine and polyamine content was performed the samples were stored at -18°C . The separation and quantitative estimation of the diamines and polyamines were carried out by using the automatic amino acid analyzer LKB-BIO-CAL 3201. The method as described by Henningsson (1978) was used with minor modifications.

The diamine and polyamine content in the ovaries of rats injected with 50 IU of hCG was also estimated after pre-treatment of the animals with the diamine oxidase inhibitor aminoguanidine (Table 3). Ten mg aminoguanidine hemi-sulphate/kg was given subcutaneously daily from 26 days of age. The last injection of this inhibitor was given half an hour before the administration of hCG.

Assay of diamines and polyamines in the urine. The urine of rats injected with 50 IU of hCG was assayed for its content of cadaverine, putrescine, spermidine and sper-

mine. Before collection of urine the rats were accustomed to the metabolism cages which provided facilities for collection of urine and faeces separately. The rats were pre-treated with aminoguanidine as described above. Immediately after the hormone injection the rat was placed in the metabolism cage and urine was collected for 24 h. Hydrochloric acid, sufficient to give the urine a pH value below 2, was added to the collection vessel to prevent formation and/or destruction of the amines by bacteria during the 24 h collection period. After rinsing the collecting equipment with distilled water the volume of the sample was noted and the urine was filtered through a Munktell's filter paper No. V2OH. The urine was then stored at -18°C until an aliquot was prepared for analysis of its amine content. To each ml of urine 1 mg EDTA and 30 mg sulphosalicylic acid were added. After centrifugation at $800 \times g$ for 15 min the subsequent preparation and assay were carried out as described above for content of diamines and polyamines in ovaries.

Results

Formation of cadaverine and putrescine in the ovaries. The in vitro formation of cadaverine and putrescine was determined in the ovaries of rats at various intervals after the administration of 50 IU of hCG. As the values of diamine formation in the control ovaries did not vary with time (1–12 h) after the injection of saline they were brought together and presented as one mean value (Fig. 1). As early as two h after the hormone injection the formation of cadaverine and putrescine was significantly increased ($P < 0.05$ and $P < 0.01$, respectively). Peak values of both cadaverine and putrescine formation were obtained 5 h after the hormone injection. The maximum formation of cadaverine was 15 times the controls and the maximum formation of putrescine 160 times the control values. At 12 h the formation of both cadaverine and putrescine was considerably lower, but had not reached their control values. Stoichiometric determinations of the formation of cadaverine in the ovary of the pregnant rat have shown a fair accordance (Andersson et al. 1979).

The formation of cadaverine and putrescine in response to different doses of hCG is presented in Table 1. Maximum diamine formation was obtained in the dose range of 25 to 50 IU of hCG. Doses above 50 IU were less effective.

Content of diamines and polyamines in the ovaries. The concentration of cadaverine, putrescine, spermidine and spermine was determined in the ovaries

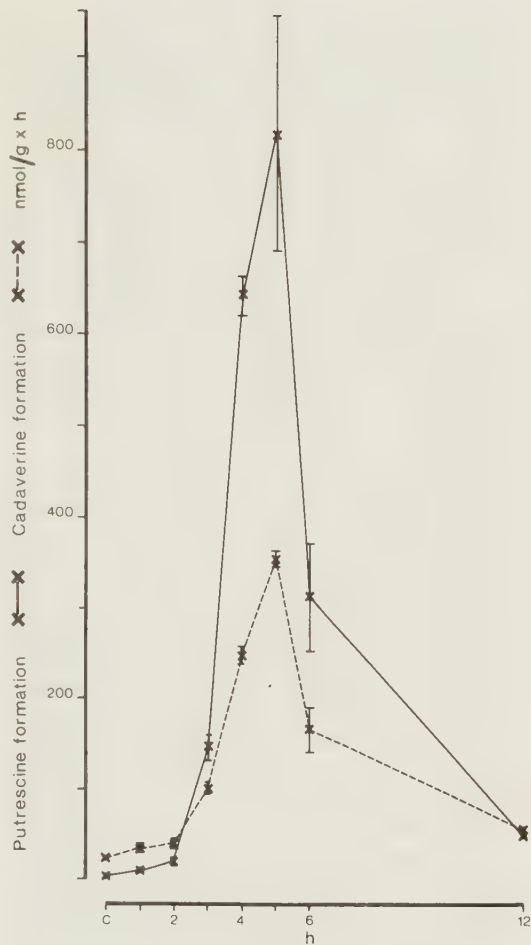


Fig. 1.

Formation of cadaverine and putrescine (nmol/g $\times$ h) in the ovaries of rats injected with hCG (50 IU) at 28 days of age. The rats were sacrificed at different times after the injection. Control rats (C) received saline. Each point represents the mean value $\pm$ SE of mean.

Controls: $n = 7$, hCG-treated: $n = 4$.

of rats at different times after the administration of 50 IU of hCG. From Table 2 it can be seen that after the administration of hCG the content of the diamines increased, while there was a decrease in the level of the polyamines. The increase in putrescine content reached a maximum concentration (1200–1300 nmol/g) ten times the control level 5–6 h after the injection of the hormone. The concentration of cadaverine in control ovaries was not measurable. Five h after hCG administration it

Table 1.

Formation of cadaverine and putrescine (nmol/g $\times$ h) in the ovaries of 28-day-old rats injected with different doses of hCG 5 h prior to the sacrifice of the animals. The figures are mean values $\pm$ SE of mean of four determinations

IU of hCG	Cadaverine formation	Putrescine formation
0	36 $\pm$ 6.71	26 $\pm$ 9.51
1.5	75 $\pm$ 13.9	82 $\pm$ 31.3
5	170 $\pm$ 20.5	400 $\pm$ 92.0
15	290 $\pm$ 12.5	790 $\pm$ 153
25	390 $\pm$ 20.1	1300 $\pm$ 124
35	370 $\pm$ 22.4	1200 $\pm$ 209
50	350 $\pm$ 33.3	1100 $\pm$ 112
75	290 $\pm$ 17.0	960 $\pm$ 169
100	270 $\pm$ 20.2	660 $\pm$ 34.3
200	280 $\pm$ 19.6	810 $\pm$ 64.4

reached a maximum value of 25 nmol/g. The decrease in the level of the polyamines displayed different courses. While there was a fairly gradual decrease in the spermine content with a maximum reduction of 44% 12 h after the hormone administration, the reduction of spermidine was about 15% 2–5 h after the hormone administration and the control level was resumed and even slightly enhanced after 12 h.

Treatment of the rats with aminoguanidine elevated the content of cadaverine in the ovary, while putrescine and polyamine content remained essentially unchanged in both control and hCG treated rats (Table 3).

Urinary excretion of diamines and polyamines. The urinary excretion of cadaverine, putrescine, spermidine and spermine was determined in urine collected for 24 h after the injection of 50 IU of hCG (Table 4). The urinary excretion of spermidine was significantly increased in the hormone treated rats, while there was a slight but not significant increase in the urinary excretion of cadaverine, putrescine and spermine. Hydrolysis of urine from adult female rats has been shown to cause a moderate increase in the amine content of the urine without changing the general pattern of the excretion of diamines and polyamines (Andersson et al. 1978a).

Discussion

Until recently nothing had been disclosed on the formation of cadaverine in mammalian tissue. Most authors have regarded cadaverine as a compound formed by bacteria and plants (Boeker & Snell 1972; Ambe & Sohoni 1959). However, the present report, together with earlier findings (Henningson et al. 1976; Andersson et al. 1979) supports our view that cadaverine can be formed *in vivo* by mammalian tissue cells and is then not a product of bacterial decarboxylation of lysine.

Regarding putrescine, the conspicuous stimulation of the formation of this diamine in the ovary in the evening of pro-oestrus, i.e. between the surge of LH and ovulation, appeared to be closely related to the ovulatory process (Kobayashi et al. 1971). The present study suggests that cadaverine formation might also be involved in this process.

Table 2.

Content of diamines and polyamines (nmol/g) in the ovaries of rats injected with hCG (50 IU) at 28 days of age. The rats were sacrificed at different times after the injections. Control rats received saline. The figures are mean values $\pm$ SE of mean. $n = 7$ (control rats), $n = 4$ (hCG-treated rats). 0 stands for not measurable amount.

Hours after injection	Cadaverine	Putrescine	Spermidine	Spermine
Saline, 1–12 h	0	120 $\pm$ 15.0	1100 $\pm$ 19.9	1000 $\pm$ 39.2
hCG, 1 h	4.4 $\pm$ 2.94	120 $\pm$ 9.92	1200 $\pm$ 102	920 $\pm$ 17.2
hCG, 2 h	4.4 $\pm$ 3.14	130 $\pm$ 15.9	940 $\pm$ 35.8**	790 $\pm$ 24.3**
hCG, 3 h	9.6 $\pm$ 5.58*	270 $\pm$ 12.6***	960 $\pm$ 22.2***	780 $\pm$ 30.5***
hCG, 4 h	20 $\pm$ 3.32***	740 $\pm$ 29.7***	980 $\pm$ 37.2**	840 $\pm$ 44.9*
hCG, 5 h	25 $\pm$ 9.50**	1200 $\pm$ 78.4***	940 $\pm$ 59.4**	730 $\pm$ 22.9***
hCG, 6 h	15 $\pm$ 8.83*	1300 $\pm$ 52.0***	1000 $\pm$ 39.0*	760 $\pm$ 32.4***
hCG, 12 h	3.6 $\pm$ 3.56	700 $\pm$ 47.2***	1200 $\pm$ 66.7*	640 $\pm$ 42.3***

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ versus controls.

Table 3.

Content of diamines and polyamines (nmol/g) in hCG stimulated (50 IU of hCG) ovaries of rats pre-treated with aminoguanidine. The rats were sacrificed at different times after the injections. Controls received saline. The figures are mean values $\pm$ SE of mean of four determinations.

Hours after injection	Cadaverine	Putrescine	Spermidine	Spermine
Saline, 5–12 h	9.5 $\pm$ 5.47	99 $\pm$ 7.89	1100 $\pm$ 81.6	970 $\pm$ 83.7
hCG, 5 h	38 $\pm$ 8.74*	1000 $\pm$ 123***	900 $\pm$ 19.9*	650 $\pm$ 27.4**
hCG, 6 h	33 $\pm$ 3.12**	1300 $\pm$ 110***	1000 $\pm$ 20.7	740 $\pm$ 35.4*
hCG, 12 h	23 $\pm$ 3.13*	730 $\pm$ 62.3***	1300 $\pm$ 30.5*	680 $\pm$ 51.7*

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ versus controls.

The elevated diamine formation following hCG administration was paralleled by and increased endogenous content of cadaverine and putrescine in the ovary. The increased content of these amines following gonadotrophin stimulation indicates that they can be formed *in vivo* by the decarboxylation of the relevant amino acids, lysine and ornithine, respectively.

It should be noted that hCG treatment decreased the tissue concentration of spermidine and spermine in the ovary. In rat prostate and in Ehrlich ascites cells putrescine has been shown to be a competitive inhibitor of spermine synthesis (Pegg & Williams-Ashman 1970; Kallio et al. 1977). Cadaverine has similarly been found to act as a competitive inhibitor of the synthesis of spermidine (Kallio et al. 1977). Thus the increased concentrations of the diamines in the ovary of the rat stimulated with hCG might influence the content of spermidine and spermine.

Treatment of the animals with aminoguanidine elevated the content of cadaverine but not that of the other amines studied. Cadaverine has been

considered to be the best substrate of diamine oxidase (Bardsley et al. 1970) and with respect to the recent finding of diamine oxidase activity in the ovary of the pregnant rat (Andersson & Henningsson 1980), this enzyme may function as a regulator of the intra-ovary level of cadaverine. In several tissues of the pregnant rat the content of cadaverine has been shown to be increased after the administration of aminoguanidine (Andersson et al. 1979).

A clear dose-response relation was seen after *in vivo* stimulation of cadaverine and putrescine formation by hCG; a rather low dose of the hormone was sufficient to produce a metabolic response which suggests a close association of diamine synthesis and the level of the gonadotrophin. In fact, a bioassay of LH based upon the *in vivo* induction of ovarian ornithine decarboxylase activity in the rat has recently been developed (Nureddin 1977).

In most mammalian tissues the diamine putrescine is considered to serve as a precursor of the polyamines, compounds with a postulated role in growth processes (for ref. see Tabor & Tabor

Table 4.

Urinary secretion of diamines and polyamines (nmol/24 h) in rats treated with hCG (50 IU) at 28 days of age. Control rats received saline. All rats received aminoguanidine from 26 days of age. The figures are mean values $\pm$ SE of mean of six determinations.

Treatment	Cadaverine	Putrescine	Spermidine	Spermine
Saline	960 $\pm$ 173	4900 $\pm$ 615	540 $\pm$ 129	13 $\pm$ 2.54
hCG	1400 $\pm$ 221	5900 $\pm$ 389	1100 $\pm$ 227*	15 $\pm$ 3.87

* = $P < 0.05$ versus controls.

1976; Jänne et al. 1978). The development of a new approach of measuring diamine oxidase activity (Andersson et al. 1978b) made possible the recent finding of diamine oxidase activity in the rat ovary (Andersson & Henningsson 1980; Andersson et al. 1980) which was earlier considered to be a tissue devoid of diamine oxidase activity (Guha & Jänne 1976). Our present findings support the hypothesis that cadaverine and putrescine themselves or products of their oxidation e.g. δ -aminovalerianic acid and γ -aminobutyric acid (GABA) may be functionally important. Formation of GABA via oxidative deamination of putrescine has recently been shown in the pregnant rat and in the gonadotrophin stimulated ovary of the pre-pubertal rat (Andersson & Henningsson 1980; Andersson et al. 1980). Notably GABA has been found to stimulate protein synthesis (Baxter 1970) and may thus be of importance in various physiological connections.

That cadaverine formation in the mouse kidney stimulated to growth by anabolic steroids may be mediated via the decarboxylation of lysine by ornithine decarboxylase was originally suggested by Henningsson et al. (1976). Recently this hypothesis has gained confirmation and extension as well (Persson 1977; Pegg & McGill 1979). However, purified ornithine decarboxylase from regenerating rat liver and liver of rats treated with thioacetamide was not inhibited by high concentrations of L-lysine indicating that the amino acid was not a substrate of ornithine decarboxylase (Raina & Jänne 1968; Williams-Ashman et al. 1969; Ono et al. 1972). The existence of multiple forms of ornithine decarboxylase which may catalyze different reactions or be of importance in ornithine decarboxylase activity regulation, has been reported by several groups (Obenrader & Prouty 1977; Fuller et al. 1978; Mitchell et al. 1978). In the pre-pubertal rat ovary, a differential stimulation of cadaverine and putrescine formation by hCG and pregnant mare serum gonadotrophin could be quantified (unpublished results). Further use of specific inhibitors of ornithine decarboxylase, such as α -methyl-ornithine (Abdel-Monem et al. 1974) and α -difluoromethyl-ornithine (Metcalf et al. 1978) and kinetic analysis of the activity of the enzyme(s) might throw additional light on these questions. The use of specific inhibitors of the appropriate enzyme(s) decarboxylating lysine and ornithine could also be useful tools in investigating a possible influence of inhibited diamine formation

on ovarian function. In fact, preliminary experiments suggest that α -difluoromethyl-ornithine may interfere with this function in rats.

The present results indicate that the ovary stimulated by hCG, in addition to putrescine, forms cadaverine at a highly increased rate. The elevated diamine formation is consistent with an increased amine content in the ovary. It should be noted that in microorganisms cadaverine has been suggested to possess some of the biological activity of putrescine and spermidine, perhaps via the formation of a spermidine analog (Dion & Cohen 1972).

The present results further indicate that parallel studies of the metabolism of cadaverine and putrescine are desirable, especially with regard to a possible differential function of these amines in the ovariatory process. In addition this study confirms the earlier finding in the mouse kidney that cadaverine can be synthesized in an inducible manner in mammalian tissue.

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Aspects on diamine oxidase activity and its determination

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Abstract

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Homogenate of guinea-pig liver and human placenta, tissues known to be rich in diamine oxidase, were incubated with ^{14}C -putrescine and the metabolites formed were determined. In the incubate of guinea-pig liver, the major metabolites were GABA and some unidentified compound(s); Δ^1 -pyrroline and $^{14}\text{CO}_2$ were also obtained. In both the maternal and fetal parts of human placenta, radioactive GABA and the unidentified compound(s) as well as Δ^1 -pyrroline were found. The results indicate that GABA is an important intermediate in putrescine metabolism. Determination of the amount of Δ^1 -pyrroline is thus not well suited as a measure of the diamine oxidase activity in tissues.

Key words: Diamine oxidase activity, putrescine metabolism, GABA, Δ^1 -pyrroline

Diamine oxidase (EC 1.4.3.6) catalyses the oxidation of diamines such as histamine, putrescine and cadaverine, yielding in the process an aminoaldehyde. The enzyme was thought to attack principally histamine and for that reason was named histaminase. It was later shown that putrescine and cadaverine are the preferred substrates (for ref. see Zeller 1965, Bardsley *et al.* 1970).

It is well established that when putrescine is incubated in the presence of purified diamine oxidase γ -aminobutyraldehyde is formed which almost quantitatively is transformed into the cyclic compound Δ^1 -pyrroline. The in vitro measurement of diamine oxidase activity according to Okuyama and Kobayashi (1961) is in reality based upon the determination of ^{14}C - Δ^1 -pyrroline formed from added ^{14}C -putrescine to the incubate. The Δ^1 -pyrroline is extracted into a toluene liquid scintillation mixture for assay of the radioactivity. This method is widely used, mostly for measurement of diamine oxidase activity in plasma. The assay is also used in the determination of diamine oxidase activity in tissues where the method has not been well characterized.

It has been shown that injection of ^{14}C -putrescine was followed by the expiring of large amounts of radioactive CO_2 within a couple of hours and γ -aminobutyric acid (GABA) was found to be an intermediate in the catabolism of putrescine to carbon dioxide (Seiler and

Eichentopf 1975, Henningsson and Rosengren 1976). The catabolism of ^{14}C -putrescine to radioactive GABA and CO_2 was blocked by aminoguanidine, a specific diamine oxidase inhibitor.

The aim of the present study was to determine the nature of the metabolic products formed from putrescine via the diamine oxidase pathway on incubating tissues *in vitro* and to develop another approach to the assay of diamine oxidase activity in tissues taking into consideration the main products formed in the incubate, thus applying our *in vivo* findings of putrescine catabolism on *in vitro* studies. A preliminary report of this work has been presented elsewhere (Andersson *et al.* 1977).

Methods

Adult female guinea-pigs, weight 830–950 g, were used. They were fed a standard pellet diet and tap water *ad libitum*. The animals were stunned and exsanguinated whereafter pieces from the liver lobes were cut out immediately. The tissue was cooled on ice, minced with scissors, weighed and gently homogenized in a Dounce type homogenizer (25 strokes with the pestle) in 4 volumes of 0.1 M cold sodium phosphate buffer pH 7.2 containing 0.2% (w/v) glucose. The homogenate was used as enzyme source.

Human placenta was obtained at term and was divided into its maternal and fetal parts. The maternal part was taken from the outermost layer and the fetal part from a layer at a depth of about 1 cm from the surface. Homogenate was prepared in the same way as above.

Incubation procedure. The incubation mixture contained homogenate corresponding to 20, 80 or 160 mg of tissue, 1,4- ^{14}C -putrescine (final conc. 10^{-4} M, sp.act. 0.125 mCi/mmol) and the same buffer as used for homogenization, the total finally made up to a volume of 1.5 ml. The mixture was incubated for different times at 37°C. Blanks were provided by preincubation with 100 μl aminoguanidine hemisulphate (final conc. 2×10^{-5} M). The incubation was stopped by tipping 0.7 ml 2 M hydrochloric acid (samples subjected to thin-layer electrophoresis) or 0.7 ml 12.6% (w/v) sulfosalicylic acid (samples subjected to automated liquid chromatography) into the incubate from a side arm of the incubation vessel. A further 45 min shaking followed for complete absorption of expelled $^{14}\text{CO}_2$ (see below).

Determination of radioactive substances resulting from catabolism of ^{14}C -putrescine. The following ^{14}C -putrescine metabolites were determined: CO_2 , GABA, Δ^1 -pyrroline and, in addition, some unidentified compound(s). Each of these products (with the exception of $^{14}\text{CO}_2$) was measured in two different systems. Some incubates were used for assays by automated liquid chromatography. Parallel incubates were divided into two parts, where one was used for extraction into toluene, the other for thin-layer electrophoresis. Duplicates were performed of all determinations with the exception for assays carried out with automated liquid chromatography. Thin-layer electrophoresis and automated liquid chromatography were also used for determination of the amount of unmetabolized labelled putrescine at the end of incubation.

1. Expelled $^{14}\text{CO}_2$ was trapped on a 10×25 mm piece of No. 005 Munktell's filter paper prepared with 100 μl hydroxide of Hyamine 10-X. After termination of the incubation the filter paper was dropped into 8 ml of Bray scintillation mixture (Bray 1960) and the radioactivity was measured in a liquid scintillation spectrometer (Packard TRI-CARB, 2002).

2. Chromatographic separation of GABA was carried out using an automatic amino acid analyzer (LKB-BIOCAL 3201). Application of liquid chromatography in diamine research has been described by Hatano *et al.* (1970). The procedure as carried out in our laboratory is as follows. After centrifugation and adjusting of pH to 2.0–2.5 with NaOH, the incubate was filtered through a filter with a pore size of 0.22 μm (Millipore corp., Bedford, Mass. 01730). The radioactive compounds of the incubate were separated at 60°C on a column of Durram DC 6A. In separating amino acids and amines, four buffers with different pH and varying amounts of sodium citrate and sodium chloride were pumped through the column in sequence at a flow-rate of 80 ml/h for different elution times: Buff. A: 0.20 M Na^+ , pH 3.50, 44 min; Buff. B: 0.35 M Na^+ , pH 6.30, 44 min; Buff. C: 0.90 M Na^+ , pH 6.20, 47 min; Buff. D: 2.80 M Na^+ , pH 5.90, 40 min. After passing the column the stream was split according to Lou (1973). One half proceeded to a fraction collector by means of which 2 ml fractions could be collected throughout the analysis. Of these fractions an aliquot of 0.5 ml was added to 3 ml Instagel (Packard) scintillation solution and the radioactivity was measured. The other half of the column eluate was mixed with ninhydrin and peaks were produced by ninhydrin-positive sub-

stances in the regular way. The final identification of the compounds in relevant fractions of eluates from the amino acid analyzer has been described (Henningsson and Rosengren 1976).

In addition radioactive GABA was estimated by thin-layer electrophoresis. Aliquots of the incubate were centrifuged at $80 \times g$ for 20 min. Of the supernatant $250 \mu\text{l}$ was added to $5 \mu\text{l}$ of a standard carrier solution ($1 \mu\text{g}$ unlabelled GABA and $1 \mu\text{g}$ unlabelled putrescine in $5 \mu\text{l}$) and 2.5 ml ethanol completed the precipitation of proteins. The samples were stored at -18°C until electrophoresis was performed. Next, the mixture was centrifuged, the supernatant was evaporated to dryness, the residue was resolved in $25 \mu\text{l}$ of 70% ethanol and quantitatively applied to a thin-layer plate (TLC plates silica gel 60, Merck). The thin-layer electrophoresis which separated the radioactive compounds was performed at 20 V/cm for 70 min and about 3°C using pyridine-acetate buffer pH 4.8 (Fischer and Bohn 1957). The plate was dried and the relevant fractions were removed in zones. The silica gel powder was transferred to vials containing 10 ml scintillation solution (5.5 g Permablend III, Packard, in one litre of toluene) and the radioactivity was measured.

3. The procedure employed for determination of Δ^1 -pyrroline was the method devised by Okuyama and Kobayashi (1961). Kusche *et al.* (1973) have given a detailed description of the procedure. Aliquots of the incubate were mixed with saturated NaHCO_3 and NaOH solution to obtain a pH of 9.5 ± 0.1 . Two ml of this sample was transferred to a test tube containing 6 ml cold toluene. The test tube, equipped with a glass stopper, was vigorously shaken for 4 min, centrifuged at $900 \times g$ for 2 min after which the aqueous phase was frozen at -20 to -30°C in a bath of ethanol and solid carbon dioxide. Four ml of the upper toluene phase was transferred to vials containing 4 ml scintillation solution (11 g Permablend III, Packard, in one litre of toluene). The radioactivity was measured.

Determination of radioactive Δ^1 -pyrroline was also carried out by the automatic amino acid analyzer as described above. In identifying Δ^1 -pyrroline, a standard test solution was prepared by incubating ^{14}C -putrescine with purified diamine oxidase (hog kidney, Grade II, Sigma).

4. A hitherto unidentified radioactive compound(s) appeared in the samples and was determined by either of the two methods, *i.e.* the automated liquid chromatography or the thin-layer electrophoresis.

Results

In initial experiments it was shown that on incubating ^{14}C -putrescine with crude liver homogenate $^{14}\text{CO}_2$ as well as ^{14}C - Δ^1 -pyrroline were formed while at incubation with the supernatant of the liver homogenate only pyrroline was formed (Table I). These expts. also showed that the catabolism of putrescine was considerably higher in the homogenate than in the supernatant. The results encouraged the search for intermediate metabolites of radioactive putrescine. In these expts. homogenate of guinea-pig liver was incubated with ^{14}C -putrescine for 1 h. The putrescine metabolites, as well as the residue of added putrescine, were determined in either of two systems. The results are seen in Table II. Except for the unknown compound(s), a good agreement was obtained by the methods used. The discrepancy regarding the unknown compound(s) is the subject of further investigation. Using the automated liquid chromatographic method a split-stream technique is necessary to determine radioactive compounds, a technique which is rather expensive and laborious. For that reason we henceforth in this study refer to results obtained by the toluene extraction of pyrroline and by thin-layer electrophoresis of the other putrescine derivatives.

From Table II it appears that an inverse relation existed between on the one hand the amount of unmetabolized putrescine and, on the other, the amount of GABA and the unknown compound(s), *i.e.* the more substrate used up the more GABA and unknown compound(s) were formed. Regarding pyrroline no such relation was found.

Experiments were performed to demonstrate the relationship between the rate of formation of the different putrescine metabolites and the amount of tissue in the incubate. Aliquots

TABLE I. Formation (nmol/g $\times$ h) of radioactive Δ^1 -pyrroline and CO_2 from ^{14}C -putrescine in homogenate (H) and supernatant (S) of guinea-pig liver in 3 experiments. The sum of the two products is also given.

Δ^1 -pyrroline		CO_2		Δ^1 -pyrroline plus CO_2	
H	S	H	S	H	S
84	77	410	0.255	490	78
51	30	450	0.52	500	31
61	24.2	350	7.6	410	32

of homogenate corresponding to 20, 80 and 160 mg of liver from the same animal were incubated for 1 h. The following products of ^{14}C -putrescine were noted: CO_2 , Δ^1 -pyrroline, GABA and the unknown compound(s). The major metabolites formed were GABA and the unidentified compound(s) (Fig. 1). In the three experiments referred to in Fig. 1 there was a linear relationship between tissue amount and rate of formation of these two metabolites up to 80 mg tissue per sample. With 160 mg of tissue the curve was flattening in all 3 expts. As to $^{14}\text{CO}_2$, the production of this putrescine metabolite was low, and an approximate linear relation to the tissue amount was found; this is in contrast to Δ^1 -pyrroline, the proportion of which was highest in the samples with low tissue amount. In Fig. 1 the total amount of the observed putrescine metabolites is also plotted. In 2 of the 3 expts. the oxidative deamination was high, the sum of the products constituting 21 and 18 % of the ^{14}C -putrescine substrate in samples of 20 mg, and 83 and 64 %, respectively, in samples of 160 mg (Fig. 1a and 1b). The third liver had a lower enzyme activity, the products constituting 7 % in samples with homogenate of 20 mg tissue and 24 % with 160 mg (Fig. 1c). The curve representing the sum of putrescine metabolites is approximately linear with the tissue amount of 20 and 80 mg

TABLE II. Comparison of different methods used for the assay of metabolic products [Δ^1 -pyrroline, GABA and an unknown compound(s)] of ^{14}C -putrescine and the residue of putrescine after incubation of ^{14}C -putrescine (58 000 dpm/sample) with liver homogenate. The methods used were automated liquid chromatography (ALC), toluene extraction (TE) and thin-layer electrophoresis (TLE). The figures are dpm/sample.

	Δ^1 -pyrroline		GABA		Unknown compound(s)		Putrescine	
	ALC	TE	ALC	TLE	ALC	TLE	ALC	TLE
	1 460	950	4 000	4 500	3 200	2 330	45 000	47 000
	248	410	8 900	15 600	5 300	5 900	—	29 300
	1 630	1 990	5 000	6 400	3 500	3 500	38 000	45 000
	1 460	1 350	27 100	23 400	20 400	14 100	5 300	8 500
	810	540	31 000	28 800	23 100	15 500	3 000	5 900
	1 410	1 320	6 200	5 900	5 600	3 300	48 000	46 000
	1 610	1 430	18 900	19 100	20 200	11 500	17 700	19 700
	284	540	19 900	21 900	15 600	12 000	18 800	16 600
mv	1 110	1 070	15 100	15 700	12 100	8 500	25 200	27 200
$\pm$ S.E.M.	± 206.0	± 194.9	$\pm 3\,720$	$\pm 3\,240$	$\pm 3\,020$	$\pm 1\,880$	$\pm 7\,000$	$\pm 6\,000$

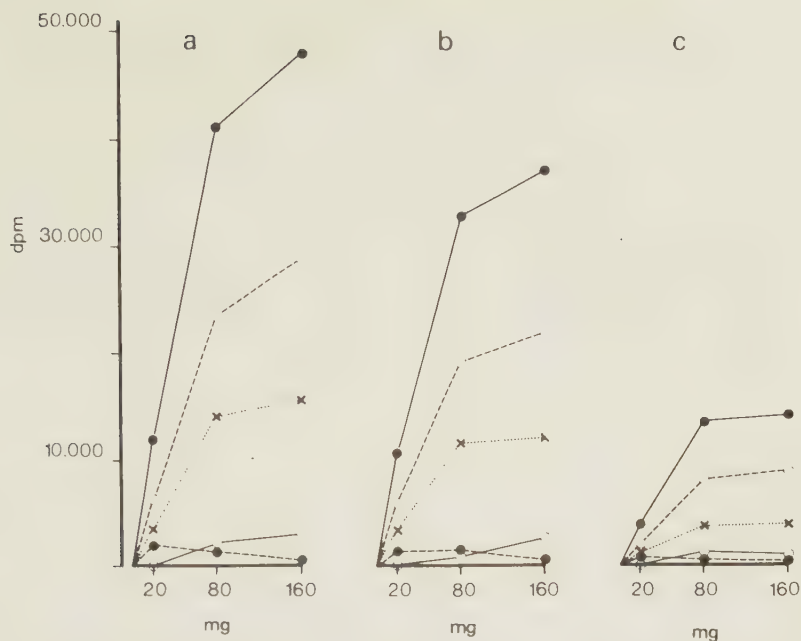


Fig. 1. Rate of formation of different metabolites from ^{14}C -putrescine in guinea-pig liver as related to the amount of tissue. The metabolites were CO_2 (○—○), Δ^1 -pyrroline (●—●), GABA (○—○) and unknown compound(s) (× ... ×). The total amount of these metabolites is also given (●—●).

but not with 160 mg, this applicable to both the expts. with high and low oxidative deamination.

The relationship between the incubation time and amount of products formed was also studied (Fig. 2). The tissue amount in the samples was 20 mg in 2 expts. and 80 mg in two others. GABA and the unknown compound(s) were again the major metabolites and the production of them was linear up to at least 1 h. With 80 mg of tissue there was a linear relation between the amount of $^{14}\text{CO}_2$ produced and the incubation time. With 20 mg of tissue the production of $^{14}\text{CO}_2$ also rose linearly with increasing incubation time, but it was negligibly small. The time curve for Δ^1 -pyrroline showed a progressively increasing course up to 120 min in samples with 20 mg tissue. However, in samples with 80 mg tissue the amount of Δ^1 -pyrroline increased up to 60 min whereafter there was a decline in the formation of Δ^1 -pyrroline. The total amount of putrescine metabolites found plotted against time followed a straight line up to 2 h in expts. with 20 mg tissue in the incubate. With 80 mg the curve was linear to only 60 min.

The oxidative deamination of ^{14}C -putrescine on incubating human placenta was also studied. 80 mg of tissue was incubated for 30 and 90 min. The degradation of ^{14}C -putrescine was approximately 10 times higher in the maternal part than in the fetal one. In both parts radioactive GABA and the unknown compound(s) were formed as well as Δ^1 -pyrroline. Formation of $^{14}\text{CO}_2$ could also be recorded. In contrast to the expts. with guinea-pig liver Δ^1 -pyrroline was the major putrescine derivative. In the maternal part the amount of pyrro-

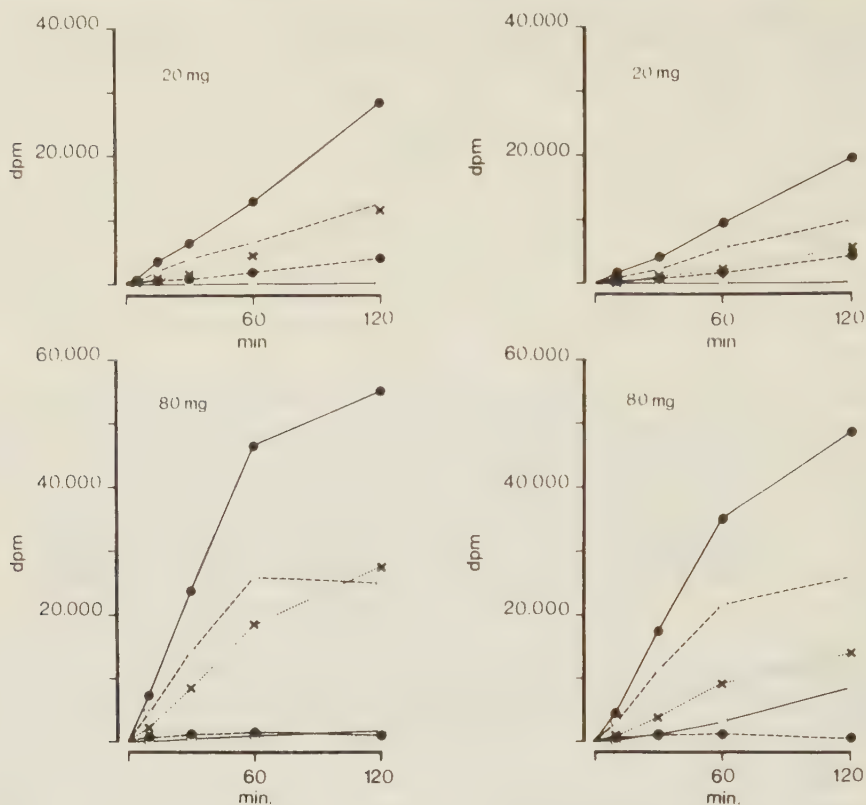


Fig. 2. Rate of formation of different metabolites from ^{14}C -putrescine in guinea-pig liver as related to the time of incubation. The metabolites were CO_2 ($\circ-\circ$), Δ^1 -pyrroline ($\bullet-\bullet$), GABA ($\square-\square$) and unknown compound(s) ($\times-\times$). The total amount of these metabolites is also given ($\bullet-\bullet$).

line formed in the samples was so high as to interfere with the determination of the other products in the thin-layer electrophoresis system. Results obtained on incubating human fetal placenta are given in Table III.

Discussion

The present *in vitro* study shows that mammalian tissues rich in diamine oxidase are capable of producing various compounds derived from putrescine; compounds which recently have been found after *in vivo* administration of putrescine (Seiler *et al.* 1971, Henningsson and Rosengren 1976). Our present work also indicates that diamine oxidase is important in the putrescine metabolism, one of the major metabolites formed being GABA. *In vivo* studies with ^{14}C -GABA have shown it to enter the tricarboxylic acid cycle (Roberts 1956, Wilson *et al.* 1959). Thus, a main route in the metabolism of putrescine seems to be a multi-step sequence with several enzymes involved where the first two steps of the route are catalyzed by diamine oxidase and γ -aminobutyraldehyde dehydrogenase, respectively. In considering the production of CO_2 as related to time of incubation, the multi-step sequence in the pathway between putrescine and CO_2 should be kept in mind.

TABLE III. Formation of CO_2 , Δ^1 -pyrroline, GABA and the unknown compound(s) from ^{14}C -putrescine in fetal human placenta. Aliquots of placental tissue homogenate were incubated for 30 and 90 min. The sum of the products is also given. The figures are dpm/sample.

CO_2		Δ^1 -pyrroline		GABA		Unknown compound(s)		Sum	
30 min	90 min	30 min	90 min	30 min	90 min	30 min	90 min	30 min	90 min
13.9	52	2 600	6 800	510	2 140	0	1 590	3 200	10 500
2.00	16.7	510	1 500	400	2 380	530	1 750	1 440	5 600
3.0	52	1 640	6 900	300	1 010	310	1 290	2 250	9 200
7.7	35	2 170	5 400	2 580	3 500	100	1 570	4 900	10 500
34	—	12 700	—	5 700	—	1 330	—	19 800	—
2.45	—	1 220	—	700	—	640	—	2 560	—

It is known that γ -aminobutyraldehyde is non-enzymatically converted into Δ^1 -pyrroline and that at steady-state the two compounds exist in equilibrium (Jakoby and Fredericks 1959). The method elaborated by Okuyama and Kobayashi (1961) measures only one of the products formed from putrescine, *i.e.* Δ^1 -pyrroline. In the present study the amount of pyrroline was found unrelated to the amount of tissue and the time of incubation. In addition in the guinea-pig liver the pyrroline represented only a small fraction of the products formed from putrescine via the diamine oxidase pathway. It thus appears that the method of Okuyama and Kobayashi (1961) should not be used indiscriminately for determination of diamine oxidase activity in tissues. This rapid method has been widely used, mostly for determination of diamine oxidase activity in plasma. It has also been used for assay of diamine oxidase activity in tissues utilizing homogenate or supernatant of tissue extract. For determination of diamine oxidase activity in plasma the method appears safe as plasma lacks enzymes which degrade γ -aminobutyraldehyde.

In the guinea-pig liver the major radioactive metabolite of putrescine found, besides GABA, was an unidentified compound(s). This is in agreement with our results after administration of ^{14}C -putrescine to mice (Henningsson and Rosengren 1976). Seiler and Eichenkopf (1975) have observed urinary excretory products of putrescine, which they suggest are spontaneous reaction products of the highly reactive γ -aminobutyraldehyde or of Δ^1 -pyrroline. The unknown compound(s) observed in our study was not extractable in toluene and was not found when ^{14}C -putrescine was incubated with purified hog kidney diamine oxidase. These facts together with the observation that the production of the unknown compound(s) was approximately proportional to the tissue amount and the time of incubation suggest to us that the unknown metabolite(s) is formed enzymatically. In preliminary expts. formation of the unknown compound(s) on incubating homogenate of guinea-pig liver with ^{14}C -GABA was negligible in spite of a high formation of $^{14}\text{CO}_2$, indicating the unknown compound(s) not being a metabolic product of GABA.

The production of GABA in relation to Δ^1 -pyrroline was lower in the human placenta than in guinea-pig liver. The reason for this is so far not known. One possibility is that the relation between diamine oxidase and γ -aminobutyraldehyde dehydrogenase may be different in the two tissues. Alternatively the dehydrogenase might be a more labile enzyme than dia-

mine oxidase. If so, the activity of the dehydrogenase might be reduced during labour. It should be noted that diamine oxidase activity in human pregnancy reaches a plateau during the 7th month of pregnancy with high values up to term (Ahlmark 1944). It has been shown in rats that the synthesis of putrescine is high in the placenta during the last trimester of gestation (Kim and Harris 1975, Guha and Jänne 1976). Much remains to be explored regarding the role of diamine oxidase activity in the placenta not to mention other tissues containing this enzyme.

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METABOLISM OF PUTRESCINE IN THE PREGNANT RAT

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ABSTRACT

The in vivo metabolism of ^{14}C -putrescine injected to rats before, during and after pregnancy was studied. Within 30 min of the administration of the isotope 9-12 % of the injected radioactivity was recorded as $^{14}\text{CO}_2$ in the expired air and after 5 h 60 % was expired. The radioactivity excreted in the urine during the first day following the ^{14}C -putrescine administration consisted of unmetabolized putrescine, γ -aminobutyric acid (GABA) and some unidentified compound(s). No radioactive polyamines were detected in the urine.

After treatment of pregnant rats with the diamine oxidase inhibitor aminoguanidine the expiration of $^{14}\text{CO}_2$ was almost completely inhibited. In the urine increased amounts of unmetabolized putrescine were excreted while the excretion of GABA and the unidentified compound(s) were decreased. In addition ^{14}C -spermidine appeared in the urine.

The in vitro metabolism of putrescine was determined by the incubation of different tissues of pregnant and non-pregnant rats with ^{14}C -putrescine. The ^{14}C -metabolites derived via the diamine oxidase pathway (Δ^1 -pyrroline, GABA, some unidentified compound(s) and carbon dioxide) varied in magnitude with the tissue investigated. GABA was found to be a main metabolite of putrescine in several tissues of the pregnant rat.

The content of putrescine and spermidine was elevated in several tissues as well as the blood on the 19th day of pregnancy in rats treated with aminoguanidine, while the content of spermine was unchanged.

Key words: Putrescine metabolism, diamine oxidase, rat pregnancy.

The occurrence of a high diamine oxidase (histaminase) activity in placenta and serum has been demonstrated both in man (Danforth & Gorham 1937, Marcou et al. 1938) and rat (Roberts & Robson 1953, Kobayashi 1964) during pregnancy. An elevated diamine oxidase activity has also been found in several other tissues of the pregnant rat (Kim & Harris 1973).

Histamine was formerly considered to be different from diamine oxidase, but they are now believed to be the same enzyme (Zeller 1965). The most well-recognized substrates for diamine oxidase are diamines such as histamine, putrescine and cadaverine. The oxidative deamination of putrescine via the diamine oxidase pathway yields γ -aminobutyraldehyde which is non-enzymatically converted into the cyclic compound Δ^1 -pyrroline. Assay of this compound has been used for determination of diamine oxidase activity (Okuyama & Kobayashi 1961).

However, in vivo studies have shown that radiolabelled putrescine administered to rats can be incorporated both into γ -aminobutyric acid (GABA) (Seiler et al. 1971, Seiler & Al-Therib 1974) and expired as $^{14}\text{CO}_2$ (Jänne 1967). In addition to these metabolites of ^{14}C -putrescine some hitherto unidentified compound(s) was found to be excreted in the urine (Seiler & Eichentopf 1975, Henningsson & Rosengren 1976). These in vivo findings led to the development of another approach in determination of diamine oxidase activity in tissues, using putrescine as substrate, taking into consideration the several metabolites formed in the incubate (Andersson et al. 1978a). In guinea-pig liver Δ^1 -pyrroline was shown to constitute only a minor fraction of the total amount of metabolites formed.

Besides being a substrate of diamine oxidase, putrescin is a precursor of the polyamines spermidine and spermine, biological molecules that have become the subject of intensive investigation. Increased intracellular concentration of the polyamines and elevated activities of their biosynthetic enzymes are among the earliest biochemical events following the application of a wide variety of growth stimuli in a broad spectrum of biological systems. Intracellular polyamine metabolism has been linked to the

metabolism of the nucleic acids. These observations have led to the proposal of a role for the polyamines in cellular growth and replication (for ref. see Tabor & Tabor 1976, Jänne et al. 1978). Interestingly, GABA has been found to stimulate protein synthesis (Tewari & Baxter 1969, Sandoval & Tapia 1975) and may thus have a function similar to that ascribed to the polyamines.

In the pregnant rat there is a highly elevated urinary excretion of histamine (Kahlson et al. 1958) as well as of putrescine, cadaverine and spermidine (Andersson et al. 1978b), indicating changes in the biosynthesis of these amines during pregnancy. Under the influence of the diamine oxidase inhibitor aminoguanidine the excreted amounts, particularly of putrescine and cadaverine, were greatly increased, indicating that oxidative deamination via diamine oxidase is a major route of catabolism of these compounds in rats (Andersson et al. 1978b, Rojansky et al. 1979). An increased diamine formation, i.e. formation of putrescine (Guha & Jänne 1976a) and cadaverine (Andersson et al. 1979a) has been shown in the ovary of the pregnant and gonadotrophin treated (Andersson & Henningsson 1980a) rat. Also, in the placenta a high putrescine (Maudsley & Kobayashi 1977) and cadaverine (Andersson et al. 1979a) formation has been found.

The reported findings of changes in both biosynthesis and degradation of putrescine in the pregnant rat led to the present study in which the metabolic fate of putrescine was investigated in vivo by measuring the expiration of $^{14}\text{CO}_2$ and the excretion of ^{14}C -metabolites in the urine after the injection of ^{14}C -putrescine to rats before, during and after pregnancy. The influence of aminoguanidine on the metabolism of ^{14}C -putrescine was also studied.

The in vitro metabolism of putrescine was determined by incubation of various tissues of pregnant and non-pregnant rats with ^{14}C -putrescine. In addition the putrescine and polyamine content of corresponding tissues and of blood was determined in pregnant and non-pregnant rats.

A minor part of this work has been reported elsewhere (Andersson & Henningsson 1980b).

METHODS

Animals

Adult female rats of the Sprague-Dawley strain, weight 220-250 g, were used. In the in vivo experiments the rats were fed a semisynthetic diet, 18-27 g (dry weight), daily. The composition of the diet has been given by Kahlson et al. (1958) and its content of diamines and polyamines by Andersson et al. (1978b). The putrescine content of the diet was estimated at 4 nmol/g. Rats used in in vitro experiments were fed a diet of commercial rat chow. All rats had free excess to tap water. Rats with a 5-day estrus cycle were used. The stage of the estrus cycle of the rats was determined by microscopic examination of vaginal smears taken in the morning and stained with methylene blue. Non-pregnant rats used were in diestrus phase. Rats meant to become pregnant were allowed to mate in the pro-estrus or early estrus phase. They were then transferred to a male for 24 h. The next morning when mating was confirmed by the finding of sperm in the vaginal smear and/or a vaginal plug was considered the first day of pregnancy. The rats gave birth to 4-14 young. They were not allowed to suckle their young.

Determination of in vivo metabolism of ^{14}C -putrescine

1,4- ^{14}C -Putrescine (2.5 μCi ; 100 μg), dissolved in 0.2 ml 0.9 % NaCl, was injected subcutaneously to rats before pregnancy. The expiration of $^{14}\text{CO}_2$ was recorded in one group of rats and the urine was collected for analysis of putrescine metabolites in another group of rats. On the 19th day of pregnancy and ten days after parturition ^{14}C -putrescine was again injected and $^{14}\text{CO}_2$ and urine were collected. In most cases the same rat was examined before, during and after pregnancy.

In rats on the 19th day of pregnancy the metabolism of ^{14}C -putrescine was also studied under the influence of the diamine oxidase inhibitor aminoguanidine (10 mg aminoguanidine hemi-sulphate/kg s.c. daily during pregnancy). On the day of experiment aminoguanidine was administered half an hour before the isotope.

Determination of expired $^{14}\text{CO}_2$. During a five hour period (10 x 30 min) immediately after the injection of ^{14}C -putrescine the rat was placed in a cylindrical plexiglass metabolism chamber with a volume of 700 ml. Before the start of the experiments the rat was accustomed to the metabolism chamber which was swept with a stream of air at a constant flow-rate of 600 ml/min. From the pregnant rats treated with aminoguanidine recordings of expired $^{14}\text{CO}_2$ were made for 5 x 30 min. The air stream was directed to a series of vials containing 5 ml of hydroxide of Hyamine 10-X (1 M solution in methanol) for quantitative absorption of the expired $^{14}\text{CO}_2$. Aliquots of the hydroxide were transferred to scintillation vials containing 8 ml of a liquid scintillation solution (Bray 1960). The radioactivity was recorded in a liquid scintillation spectrometer (Packard TRI-CARB, 2002). Corrections were made for loss of methanol during the collection periods.

Determination of urinary radioactivity. After the injection of ^{14}C -putrescine the rats were immediately placed in metabolism cages which allowed urine to be collected separately from faeces. Urine was collected in 24 h samples for three consecutive days after the injection of the isotope. Before the start of urine collection the rats were accustomed to the metabolism cages. The urine was collected in a vessel containing 5 drops of 10 M hydrochloric acid and 1 ml distilled water. After rinsing the collecting equipment with distilled water the volume of the sample was noted. The urine was then filtered through a Munktell's filter paper No. V20H and stored at -18°C until an aliquot was prepared for analysis of its radioactivity. For this preparation 1 mg EDTA (ethylene diamine tetraacetic acid) and 30 mg sulfosalicylic acid were added to each ml of urine, whereafter the urine sample was centrifuged at 800 x g for 15 min, adjusted to a pH value of 2.0-2.5 and filtered through a Millipore filter (0.22 μm). The separation and quantitative estimation of the relevant compounds in the urine were performed on a 6 x 78 mm column of Durrum DC 6A by means of an automatic amino acid analyser (LKB-

BIOCAL, 3201) using a split-stream technique as described by Lou (1973) and adopted in this laboratory with some modifications (Henningsson 1978). The identity of GABA was confirmed by rechromatography on the column using a buffer system described by Hamilton (1962) for separation of amino acids.

Determination of in vitro metabolism of ^{14}C -putrescine

Incubation procedure. The experiments were performed on the 19th day of pregnancy. Concomitantly non-pregnant rats were sacrificed. The rats were stunned and exsanguinated. The appropriate tissues were rapidly removed, cooled on ice, minced with scissors, weighed and dispersed in the incubation vessel in cold 0.1 M sodium phosphate buffer (pH 6.9) containing 5×10^{-4} M dithiothreitol, 10^{-4} M EDTA and 0.2 % (w/v) glucose. Pyridoxal-5-phosphate (final conc. 10^{-4} M) and 1,4- (^{14}C) -putrescine (final conc. 10^{-4} M, sp. act. 1.25 mCi/mmol) were added. About 20 mg (uterus of pregnant rats, maternal placenta and distal ileum) or 60-80 mg (uterus of non-pregnant rats, ovary, fetal placenta, liver and kidney) of minced tissue were incubated at 37°C for 15 and 60 min, respectively. Blanks were provided by preincubation with aminoguanidine hemi-sulphate (final conc. 2×10^{-5} M). The incubation was terminated by the tipping of 0.7 ml 12.6 % sulfosalicylic acid into the incubate from a side arm of the incubation vessel and expelled $^{14}\text{CO}_2$ was trapped on a 10 x 25 mm piece of Munktell's filter paper No. 005 prepared with 100 μl of hydroxide of Hyamine 10-X. For the preparation of the incubates for analysis of its radioactivity 0.4 mg EDTA was added to each ml of incubate, whereafter they were prepared and assayed in the same way as described for urine samples.

Maternal placenta, fetal placenta and liver of the pregnant rat were also incubated in the presence of the polyamine precursor S-adenosyl-L-methionine (final conc. 10^{-4} M).

Determination of content of putrescine and polyamines in tissues and blood

The content of putrescine, spermidine and spermine was determined in tissues of untreated and aminoguanidine-

-treated rats on the 19th day of pregnancy as well as in aminoguanidine-treated non-pregnant rats. The tissues (about 200 mg) were rapidly removed and immediately homogenized in 3.5 ml sulfosalicylic acid (40 mg sulfosalicylic acid and 0.4 mg EDTA/ml) in a Duran type of homogenizer. Blood was withdrawn from rats after cutting of the carotid arteries. The blood was collected in a cooled beaker prepared with heparin and aminoguanidine. To each ml of blood 0.5 ml sulfosalicylic acid (60 mg sulfosalicylic acid and 0.6 mg EDTA/ml) was added and the samples were carefully pestled. The extracts were then boiled in a water-bath for 30 min. The subsequent preparation and separation were similar to that described above for urine samples and incubates. The quantitative estimation was carried out after coupling of the amines to ninhydrin. The method described by Henningsson (1978) was used with minor modifications.

Statistics

Student's t-test for unpaired or paired observations was used. The 0.05 level of probability was accepted as significant.

RESULTS

Expiration of $^{14}\text{CO}_2$ after the injection of ^{14}C -putrescine
Radiolabelled putrescine (2.5 μCi ; 100 μg) was injected to untreated rats at three different occasions, i.e. to rats before pregnancy, on the 19th day of pregnancy and ten days after parturition. Radiorespirometric recordings of $^{14}\text{CO}_2$ were made for 5 h after the injection of ^{14}C -putrescine. The results are shown in Fig. 1A. The putrescine was rapidly metabolized; within 30 min of the administration of the isotope 9 % of the injected radioactivity was recovered as $^{14}\text{CO}_2$ in non-pregnant rats before and after pregnancy. During pregnancy this fraction was significantly increased, constituting 12 % of the injected radioactivity. However, during the whole period of 5 h there was no significant increase in the $^{14}\text{CO}_2$ excretion of the pregnant rats. Forty eight % of the given radioactivity

was expired within 2.5 h and 60 % within 5 h of the injection of ^{14}C -putrescine.

Administration of aminoguanidine almost completely inhibited the excretion of $^{14}\text{CO}_2$ (Fig. 1 B). In the pregnant rats treated with the inhibitor 0.8 % of the injected radioactivity was recovered as $^{14}\text{CO}_2$ during the 2.5 h recording period.

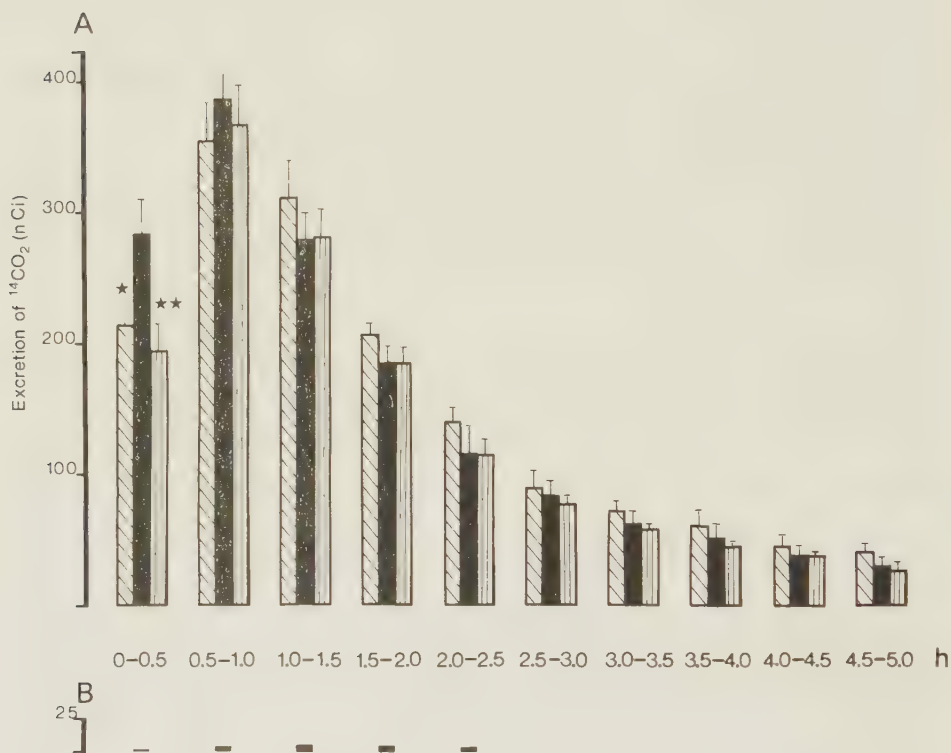


Fig. 1. Excretion of $^{14}\text{CO}_2$ (nCi) after the injection of ^{14}C -putrescine (2.5 μCi ; 100 μg) to rats. ▨ : rats before pregnancy, ■ : rats on the 19th day of pregnancy, □ : rats 10 days after delivery. A: untreated rats, B: rats treated with aminoguanidine. The columns are mean values $\pm$ S.E. of mean. Untreated rats: n=4, rats treated with aminoguanidine: n=2. * = $p < 0.05$, ** = $p < 0.01$ versus pregnant rats.

Radioactivity in the urine of rats injected with ^{14}C -putrescine

^{14}C -Putrescine (2.5 μCi ; 100 μg) was injected to untreated rats before, during and after pregnancy and to pregnant

rats treated with aminoguanidine. Urine was collected for three consecutive days following the ^{14}C -putrescine administration.

Table 1. Total radioactivity (nCi/24 h) in the urine collected for three days after the injection of ^{14}C -putrescine (2.5 μCi ; 100 μg s.c.) to rats before, during and after pregnancy. A: untreated rats, B: rats treated with aminoguanidine. The figures are mean values $\pm$ S.E. of mean. Untreated rats: n=6, aminoguanidine-treated rats n=3.

Day No.	Non-pregnant	Pregnant	Post-partum	Pregnant
	A	A	A	B
1	530 $\pm$ 49.4 **	390 $\pm$ 35.3	450 $\pm$ 44.0	1800 $\pm$ 43.3 ***
2	14 $\pm$ 0.57 *	10 $\pm$ 0.62	13 $\pm$ 0.47 *	41 $\pm$ 0.56 ***
3	8.6 $\pm$ 0.05 *	6.7 $\pm$ 0.28	8.4 $\pm$ 0.62 *	28 $\pm$ 1.27 ***

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ versus untreated pregnant rats.

The total radioactivity found in the 24 h urine samples is shown in Table 1. During pregnancy the rats excreted 16 % of the administered radioactivity in the urine, while 19 % and 22 % of the given radioactivity were found in the urine collected from rats before and after pregnancy, respectively. Treatment of pregnant rats with aminoguanidine highly elevated the excretion of urinary radioactivity; 73 % of the injected dose was recorded in their urine.

The major part (96 %) of the urinary radioactivity was excreted during the first day following administration of ^{14}C -putrescine. Fig. 2 depicts the distribution of this radioactivity on various ^{14}C -metabolites. In untreated rats the following ^{14}C -products were excreted: unmetabolized putrescine, GABA and some unidentified compound(s), the two latter being excreted in almost equal amounts both before, during and after pregnancy. ^{14}C -Putrescine was however excreted in significantly less amounts during pregnancy.

Under the influence of aminoguanidine the pregnant rats excreted 8 times more unmetabolized putrescine in the urine, while the excretion of the unidentified compound(s) and GABA were significantly decreased. In addition to the above

mentioned metabolites of ^{14}C -putrescine the aminoguanidine-treated rats excreted small amounts of ^{14}C -spermidine (Fig. 2B).

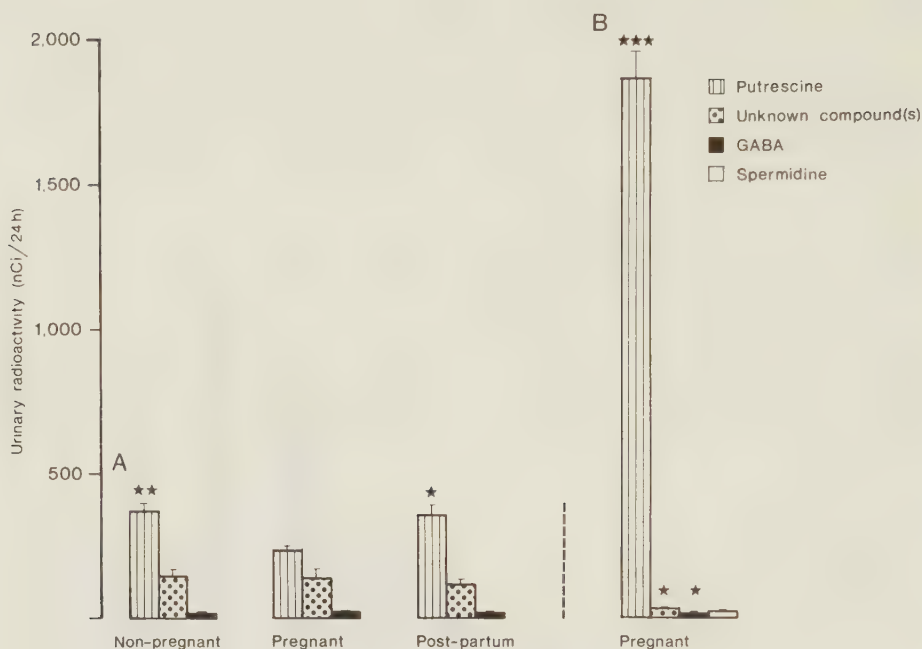


Fig. 2. Radioactive metabolites (nCi/24 h) in the urine on the first day after the injection of ^{14}C -putrescine (2.5 μCi ; 100 μg) to rats before, during and after pregnancy. A: untreated rats, B: rats treated with aminoguanidine. The columns are mean values $\pm$ S.E. of mean. Untreated rats: n=6, rats treated with aminoguanidine: n=3. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ versus pregnant untreated rats.

In vitro metabolism of ^{14}C -putrescine in rat tissues

The radioactive metabolites formed from ^{14}C -putrescine on incubating tissues from pregnant and non-pregnant rats were determined. As seen in Fig. 3 Δ^1 -pyrroline, GABA, the unknown compound(s) and carbon dioxide, metabolites of putrescine derived via the diamine oxidase pathway, were formed by all tissues of the pregnant rat examined except the kidney. The highest diamine oxidase activity per unit of weight was found in the maternal part of the placenta,

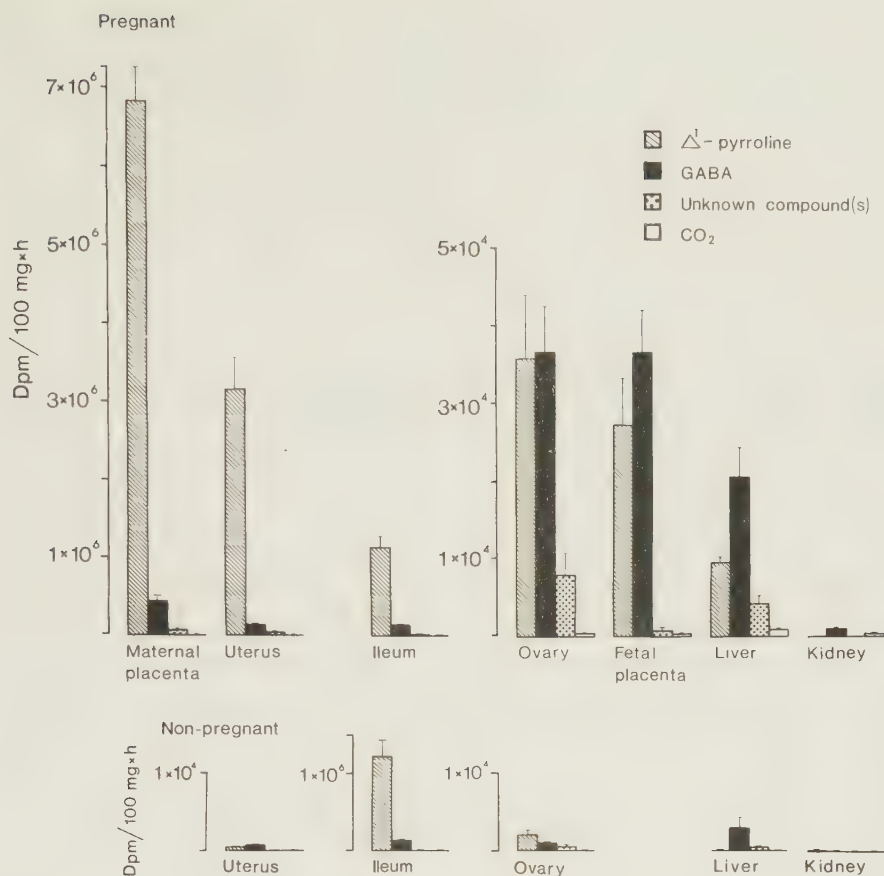


Fig. 3. Rate of in vitro formation of various metabolites from ^{14}C -putrescine in tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of gestation. Each column is the mean $\pm$ S.E. of mean of 4 determinations.

followed by the uterus and the distal ileum. The main metabolite formed from putrescine by these tissues was Δ^1 -pyrroline, in fact it constituted about 94 % of the total amount of metabolites formed. In the other tissues of the pregnant rat the pattern of metabolites was different. As seen in Fig. 3 the formation of Δ^1 -pyrroline and GABA was equal in magnitude in the ovary, while in the fetal placenta and the liver GABA was the metabolite found in the highest concentration constituting about 57 % of the total amount of metabolites formed. In the kidney the diamine oxidase activity was low; only small amounts of GABA and carbon dioxide were formed. During the experimental condi-

tions used no formation of ^{14}C -polyamines was detectable in any of the tissues investigated. The addition of S-adenosyl-L-methionine did not affect the incorporation of ^{14}C -putrescine into ^{14}C -spermidine, results comparable with the findings of Jänne (1967).

All the tissues of the non-pregnant rats examined, except the ileum where the diamine oxidase activity was of the same magnitude as in the pregnant rats, showed a low diamine oxidase activity (Fig. 3).

Content of putrescine, spermidine and spermine in tissues and blood of pregnant and non-pregnant rats

The content of putrescine in various tissues of pregnant and non-pregnant rats is shown in Table 2. In animals given aminoguanidine the concentration of this diamine was elevated in all tissues examined in the pregnant rat. The increase was most conspicuous in the ovaries where values 13 times higher than in the non-pregnant rats were obtained. Table 5 shows that the concentration of putrescine was elevated also in the blood of aminoguanidine-treated pregnant rats. Comparison of untreated and aminoguanidine-treated pregnant rats showed an elevated content of putrescine in

Table 2. Content (nmol/g) of putrescine in various tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of pregnancy. A: untreated rats, B: rats treated with aminoguanidine. The figures are mean values $\pm$ S.E. of mean, $n=4-6$.

Tissue	Pregnant	Pregnant	Non-pregnant
	A	B	B
Ovary	700 $\pm$ 53.3	690 $\pm$ 48.0	53 $\pm$ 6.10 ***
Uterus	20 $\pm$ 5.60 *	47 $\pm$ 8.25	24 $\pm$ 3.68 *
Kidney	24 $\pm$ 3.69 **	130 $\pm$ 28.5	62 $\pm$ 2.52 *
Liver	66 $\pm$ 25.2	65 $\pm$ 7.83	25 $\pm$ 4.71 **
Distal ileum	170 $\pm$ 17.9 *	240 $\pm$ 22.9	180 $\pm$ 11.3 *
Whole placenta	220 $\pm$ 22.0	180 $\pm$ 23.4	
Fetal placenta	230 $\pm$ 15.0	200 $\pm$ 16.2	
Maternal placenta	240 $\pm$ 24.4	210 $\pm$ 12.0	

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ versus pregnant rats treated with aminoguanidine.

Table 3. Content (nmol/g) of spermidine in various tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of pregnancy. A: untreated rats, B: rats treated with aminoguanidine. The figures are mean values $\pm$ S.E. of mean, n=4-6.

Tissue	Pregnant	Pregnant	Non-pregnant
	A	B	B
Ovary	1200 $\pm$ 76.3	1200 $\pm$ 43.0	800 $\pm$ 13.1 **
Uterus	630 $\pm$ 141	700 $\pm$ 66.4	580 $\pm$ 32.7
Kidney	630 $\pm$ 52.5	550 $\pm$ 46.8	490 $\pm$ 14.8
Liver	2000 $\pm$ 188	1700 $\pm$ 134	940 $\pm$ 112 **
Distal ileum	1600 $\pm$ 40.6	1700 $\pm$ 116	1400 $\pm$ 46.8
Whole placenta	960 $\pm$ 42.0	850 $\pm$ 64.8	
Fetal placenta	1100 $\pm$ 39.2 **	910 $\pm$ 27.8	
Maternal placenta	960 $\pm$ 46.5	900 $\pm$ 52.4	

** = $p < 0.01$, *** = $p < 0.001$ versus pregnant rats treated with aminoguanidine.

the blood (Table 5) and in some of the tissues investigated (Table 2). The elevation was most pronounced in the kidney where a five-fold increase was observed. One explanation of the marked increase in this organ might be correlated to the excretory function of the kidneys as the aminoguanidine-treated pregnant rat excretes extremely high amounts of putrescine in the urine (Andersson et al. 1978b).

Tables 3 and 5 show an elevated tissue and blood concentration of spermidine in the pregnant rats treated with aminoguanidine as compared to the non-pregnant ones. Administration of aminoguanidine to pregnant rats did not obviously change the spermidine content.

Except for a significant decrease in the ovary of the aminoguanidine-treated pregnant rat there were no evident differences in the content of spermine in the various tissues investigated or in the blood (Tables 4 and 5).

Table 4. Content (nmol/g) of spermine in various tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of pregnancy. A: untreated rats, B: rats treated with aminoguanidine. The figures are mean values $\pm$ S.E. of mean, n=4-6.

Tissue	Pregnant	Pregnant	Non-pregnant
	A	B	B
Ovary	610 $\pm$ 41.1	610 $\pm$ 54.1	750 $\pm$ 12.5 *
Uterus	340 $\pm$ 79.7	470 $\pm$ 86.8	310 $\pm$ 11.7
Kidney	810 $\pm$ 60.2	710 $\pm$ 62.6	790 $\pm$ 20.7
Liver	880 $\pm$ 18.1	890 $\pm$ 34.1	900 $\pm$ 34.9
Distal ileum	750 $\pm$ 48.3	700 $\pm$ 11.7	720 $\pm$ 26.2
Whole placenta	410 $\pm$ 28.0	460 $\pm$ 19.7	
Fetal placenta	430 $\pm$ 11.8	460 $\pm$ 25.7	
Maternal placenta	630 $\pm$ 45.7	550 $\pm$ 43.9	

* = $p < 0.05$ versus pregnant rats treated with aminoguanidine.

DISCUSSION

In the present report an extremely rapid in vivo catabolism of ^{14}C -putrescine in the pregnant as well as non-pregnant rat is demonstrated. The pregnant rat with its highly increased diamine oxidase activity in serum and several tissues expired significantly more $^{14}\text{CO}_2$ during the first half hour after the ^{14}C -putrescine administration. Otherwise the amount of $^{14}\text{CO}_2$ expired and the amount of ^{14}C -metabolites excreted in the urine were remarkably similar in rats before, during and after pregnancy. These results are comparable with the findings of Nilsson et al. (1959) who found no significant difference between pregnant and non-pregnant women regarding total histaminolytic activity and the pattern of ^{14}C -histamine metabolites in the urine after the injection of ^{14}C -histamine.

According to the present in vitro measurements of diamine oxidase the main metabolite formed from ^{14}C -putrescine by the maternal placenta and uterus was Δ^1 -pyrroline, indicating a low activity of aldehyde dehydrogenase in these

Table 5. Content (nmol/ml) of putrescine, spermidine and spermine in whole blood of pregnant and non-pregnant rats. The blood of the pregnant rats was collected on the 19th day of pregnancy. A: untreated rats, B: rats treated with aminoguanidine. The figures are mean values $\pm$ S.E. of mean, n=3-4.

	Pregnant	Pregnant	Non-pregnant
	A	B	B
Putrescine	7.3 $\pm$ 0.85 ***	32 $\pm$ 3.35	7.5 $\pm$ 0.91 ***
Spermidine	54 $\pm$ 5.96	50 $\pm$ 3.82	27 $\pm$ 1.07 **
Spermine	3.8 $\pm$ 0.40	3.0 $\pm$ 0.05	3.2 $\pm$ 0.28

** = $p < 0.01$, *** = $p < 0.001$ versus pregnant rats treated with aminoguanidine.

tissues. Thus, the increased diamine oxidase activity might not be paralleled by an increase in the enzymes causing the further metabolism of putrescine, a fact that might contribute to the minor differences found in the in vivo metabolism of ^{14}C -putrescine of the pregnant and non-pregnant rats. In the female rat there is an increased biosynthesis and accumulation of putrescine in the reproductive organs during pregnancy (Guha & Jänne 1976a, 1976b, Maudsley & Kobayashi 1977). The accumulation of putrescine was confirmed in the present study and was shown to be valid for some other tissues as well as the blood. Thus, the slight differences in the metabolism of ^{14}C -putrescine might also be due to a greater isotopic dilution of the ^{14}C -putrescine by unlabelled intracellular putrescine in the pregnant rat. Loading of rats with unlabelled putrescine has been shown to diminish the amount of $^{14}\text{CO}_2$ recovered during a 2 h period after the injection of ^{14}C -putrescine (Sourkes & Missala 1979). Also, the extremely rapid catabolism of ^{14}C -putrescine seen even in the non-pregnant rat probably rapidly decreases the substrate concentration of ^{14}C -putrescine. This might be an additional reason why increased expiration of $^{14}\text{CO}_2$ could be demonstrated only during the first half hour after the administration of the isotope. - 15 -

The content of putrescine in the maternal part of the placenta found in the present investigation was considerably higher than in earlier reports (Guha & Jänne 1976b, Maudsley & Kobayashi 1977). This discrepancy might depend on differences in methodology. Using the alkaline butanol extraction procedure originally elaborated for quantitation of spermidine and spermine (Raina 1963) in determinations of putrescine content a considerable part of the putrescine has been reported to be quickly destroyed (Fischer et al. 1972). Using the enzymatic-isotopic assay for measuring putrescine concentration (Harik et al. 1973), in a tissue rich in diamine oxidase, there is according to our experience, a breakdown of diamines during the preparation of the tissue extract. The same may be true, but to a smaller extent, for the polyamines as both spermidine and spermine have been shown to serve as substrates for diamine oxidase (Buffoni 1966, Bardsley et al. 1974, Hölttä et al. 1975).

The present study clearly demonstrates the importance of diamine oxidase for the in vivo metabolism of ^{14}C -putrescine in the rat. In the urine, after administration of ^{14}C -putrescine, some unknown compound(s) was shown to constitute the main metabolite. The other metabolite of putrescine found in the urine was GABA. After inhibition of diamine oxidase with aminoguanidine the expiration of $^{14}\text{CO}_2$ was almost completely inhibited and the excretion of GABA and the unidentified compound(s) was decreased while the amount of unmetabolized ^{14}C -putrescine was highly elevated. Only after the administration of aminoguanidine ^{14}C -spermidine could be quantitated in the urine indicating a preference for the metabolism of putrescine via diamine oxidase rather than conversion to polyamines.

The in vitro determinations of diamine oxidase showed, in addition to Δ^1 -pyrroline, the formation of the same ^{14}C -metabolites of putrescine as was found in the in vivo experiments. Most interesting was the finding of GABA as a major metabolite formed by the ovary, fetal placenta and liver, suggesting a possible functional significance for GABA in these organs. The pattern of ^{14}C -metabolites varied with the tissue investigated, possibly reflecting a differ-

rence in the activity of aldehyde dehydrogenase. Inhibition of aldehyde metabolizing enzymes has been shown to elevate the amount of Δ^1 -pyrroline formed in the incubate (Fogel et al. 1978) while the formation of the other metabolites of ^{14}C -putrescine was lowered (Fogel et al. 1979, Andersson et al. 1979b). Thus, these results together with earlier findings (Andersson et al. 1978a, Andersson et al. 1980a), support the view that determination of all the metabolites formed from ^{14}C -putrescine is necessary in certain tissues in order to obtain a valid measure of their diamine oxidase activity.

As to the formation of GABA, the findings in the ovary were of particular interest. This organ of the aminoguanidine-treated rats also showed large changes in putrescine and polyamine content in connection with pregnancy. The sites of GABA formation and amine synthesis in the ovary are not known and studies of isolated compartments of this organ during pregnancy and the reproductive cycle would be of interest to obtain an insight into the possible role of diamine oxidase and amine metabolism in ovarian function.

A high rate of in vitro formation of the unknown metabolite(s) of putrescine has been found after incubation of ^{14}C -putrescine with neoplastic tissue from patients with medullary carcinoma of the thyroid (Andersson et al. 1980b), a tumour associated with an elevated diamine oxidase activity. Evidence is accumulating that in patients with certain tumours and leukemia the excretion of polyamines in the urine is elevated; determination of the rate of polyamine excretion has been suggested as a biochemical test for the detection of early cancer (Russell et al. 1971). However, determination of endogenous amounts of the yet unidentified compound(s) found in our studies, in plasma and urine, could possibly become a more sensitive tool in revealing early cancer as this compound(s) seems to be the main metabolite of ^{14}C -putrescine excreted in the urine.

A recent report describes 2-pyrrolidone as a metabolite of ^{14}C -putrescine found after in vitro incubation with rat liver slices (Lundgren & Hankins 1978). However, preliminary experiments indicate that the unknown compound(s) found in our investigations might not be identical with this sub-

stance. Further studies are in progress to disclose the identity of this unidentified compound(s) in order to reveal a main metabolite of putrescine.

The marked changes in diamine oxidase activity during pregnancy excited the hope that measurements of plasma levels of the enzyme might be clinically valuable as an index of fetoplacental functionality (Borglin & Willert 1957, Beaven et al. 1975) and that fetal salvage programmes might be based on routine assays of this enzyme (Weingold & Southren 1971). Measurements of diamine oxidase activity in vaginal fluid have been shown to be a reliable method for the diagnosis of ruptured fetal membranes (Elmfors et al. 1974).

However, the biological significance of diamine oxidase is yet not settled, but a better understanding of its metabolic function may provide further insight not only to the physiological implication of this enzyme but also to that of diamines and polyamines in pregnancy as well as other biological systems.

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